

KISS1 AND ITS G PROTEIN-COUPLED RECEPTOR (GPR54)  
IN CANCER PROGRESSION AND METASTASIS

A Dissertation  
by  
SUNG GOOK CHO

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Genetics

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December 2010

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## ABSTRACT

KISS1 and Its G Protein-Coupled Receptor (GPR54) in Cancer Progression and  
Metastasis. (December 2010)

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Chair of Advisory Committee: Dr. Mingyao Liu

Activation of G-protein coupled receptor 54 (GPR54) signaling generated by kisspeptins (endogenous GPR54 ligands encoded by KISS1 gene) has been known to regulate puberty and to suppress cancer metastasis. However, an endogenous GPR54 signaling in cancer progression is still unclear. This study demonstrates that an autocrine GPR54 signaling regulates breast cancer progression and metastasis.

When MMTV-PyMT mice were crossed with Gpr54 heterozygous mice, Gpr54 heterozygosity attenuated PyMT-induced breast cancer progression, including tumorigenesis and metastasis. Likewise, Gpr54 heterozygosity retarded in vitro primary tumor cell proliferation, migration, anchorage-independent growth, and in vivo tumor growth in SCID mice. Furthermore, the anchorage-independent growth was linked to dosage-dependent Gpr54 regulation of RhoA. Human KISS1 and GPR54 were abundantly expressed in benign breast tissue. In MCF10A normal human breast epithelial cells, knockdown of GPR54 or inactivation of RhoA reduced Ras-induced anchorage-independent growth, while constitutively active RhoA recovered Ras-induced

tumorigenicity in GPR54-silenced cells. Therefore, this study suggests that autocrine GPR54 signaling via RhoA is sufficient for breast tumorigenesis.

The major population of human breast cancer is estrogen receptor-positive (ER<sup>+</sup>). This study further demonstrates that a loss of autocrine GPR54 signaling in non-metastatic ER<sup>+</sup> breast cancer cells induces estrogen-independent tumor growth and metastasis with a morphological change. In MCF7 non-metastatic ER<sup>+</sup> human breast cancer cells, loss of autocrine GPR54 signaling by knockdown of KISS1 or GPR54 caused a morphological change with an alteration of epithelial-to-mesenchymal (EMT) gene expression. Accordingly, silencing of GPR54 signaling by knockdown with KISS1 shRNA or GPR54 shRNA reduced cell proliferation, but enhanced cell motility and anchorage-independent growth. In addition, loss of autocrine GPR54 signaling caused E<sub>2</sub>-insensitivity. In xenograft tumor growth assays, the lack of autocrine GPR54 signaling caused E<sub>2</sub>-independent tumor growth. In the experimental metastasis mouse model, loss of autocrine GPR54 signaling promoted pulmonary metastasis. Thus, those data indicate that loss of autocrine GPR54 signaling causes estrogen-independent tumor growth and metastasis by promoting epithelial-to-mesenchymal transition (EMT). Altogether, this study demonstrates that the autocrine KISS1-GPR54 signaling is sufficient for breast tumorigenesis and for suppressing ER<sup>+</sup> breast cancer metastasis.

## DEDICATION

To my mother who prays for me all the time

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## CHAPTER I

### INTRODUCTION

#### 1.1 Identification and characterization of KISS1 and GPR54

KISS1 was first identified as a metastasis suppressor in metastatic melanomas and breast cancer cells (Lee et al., 1996; Lee and Welch, 1997). Earlier studies approached reversely to identify metastasis suppressor genes. Using a microcell-mediated transfer of human chromosome 6 where certain genes were supposed to be deleted in metastatic melanoma cells (C8161 or MelJuSo), Welch et al. found that it suppressed metastatic abilities in athymic nude mice (Welch et al., 1994). Later, KISS1 was identified in chromosome 6-transferred C8161 cells but not in parental C8161 cells (Lee et al., 1996), and found to suppress metastatic ability of MDA-MB-435 metastatic breast cancer cells (Lee and Welch, 1997).

Later, KISS1 was mapped at chromosome 1q32 but not chromosome 6 (Lee et al., 1996; West et al., 1998), indicating that certain genes at chromosome 6 absent in metastatic melanomas and breast cancers might regulate expression of KISS1 at chromosome 1q32. It was supported by findings that KISS1 expression was regulated by DRIP130 at chromosome 6q16.3-q23 and AP2alpha at chromosome 6p24 in metastatic melanoma cells and breast cancer cells, respectively (Goldberg et al., 2003; Mitchell et al., 2006; Mitchell et al., 2007; Williamson et al., 1996).

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This dissertation follows the style of Cancer Cell.

KISS1 gene produced kisspeptins that were posttranslationally processed, modified, cleaved, and secreted (Ohtaki et al., 2001). KISS1 protein consisting of 145 amino acids produces carboxyl-terminally amidated 54 amino acids named metastin (Ohtaki et al., 2001). Importantly, metastin suppressed pulmonary metastasis of B16-BL6 melanoma cells overexpressing G-protein coupled receptor 54 (GPR54) also called KISS1R, hOT7T175, or AXOR12 (Muir et al., 2001; Ohtaki et al., 2001). Meanwhile, metastin further produces kisspeptin-10, -13, and -14 in lengths (Ohtaki et al., 2001). That research proposed that a production of kisspeptins might follow a pro-kisspeptin release from the cell. Recently, Nash et al., however, showed that pro-kisspeptin cleavage in cells overexpressing exogenous KISS1 tagged with flag is held in cultured cells (Nash et al., 2007). Thus, a mechanism by which kisspeptin process is still unclear. Theoretically, a signal peptide from Met1 to Gly19 is removed, which follows a cleavage of pro-kisspeptin at Arg67 and at Arg124. Subsequently, Arg124 and Lys123 are removed. Finally, Phe121 is transamidated with Gly122. Therefore, the C-terminal amidation of pro-kisspeptin provides a concept that KISS1 may have a physiological function as RF-amide peptides containing RF-NH<sub>2</sub> via GPR54 (Clements et al., 2001; Kotani et al., 2001). However, biochemistry-based knowledge of KISS1 is still unclear, as RF residue of kisspeptins was not required for KISS1 function (Niida et al., 2006; Tomita et al., 2008).

GPR54, located on chromosome 19p13.3, is a member of rhodopsin family of G-protein coupled receptors (Lee et al., 1999). GPR54 was identified from rat and human brain cDNA library (Lee et al., 1999; Muir et al., 2001). Transmembrane sequences of

rat Gpr54 share 45% and 37% with rat galanin receptor and delta-1 opioid receptor, respectively, while GPR54 is specifically activated by kisspeptins ( $K_d=10\text{pmole}$ ) but not galanin receptor activators (Lee et al., 1999). Human GPR54 and mouse Gpr54 were 85% and 95% identical with rat Gpr54, respectively, and coupled to RF- or RW-amide peptides to activate  $G\alpha_q$  pathway (Clements et al., 2001). Later, mouse Kiss1 and Gpr54 were cloned from mouse brain cDNA library and revealed that those were 54% and 85% identical with human KISS1 and GPR54 (Stafford et al., 2002). In addition, human and mouse kisspeptins coupled to GPR54 induced  $\text{PLC}\beta\text{-Ca}^{2+}$  pathway and regulated cell proliferation and motility with altering the morphology (Stafford et al., 2002).

## 1.2 GPR54 signaling in cell biology

KISS1 overexpression in MDA-MB-435 cells facilitated cell spreading on type-IV collagen without affecting cell growth and motility, and further suppressed cancer metastasis (Lee and Welch, 1997). However, that study did not concern kisspeptin production as well as GPR54 existence, which indicated that KISS1 construct they used might not reflect an innate KISS1 function although they obtained expected data using it.

Later, GPR54 signaling has been found to regulate cell proliferation and motility. In CHO-K1 cells, kisspeptins derived from KISS1 via GPR54 induced phosphorylation of ERK1/2 and p38MAPK, increased arachidonic acid release, IP3 production, calcium mobilization and stress fiber formation, but inhibited cell proliferation (Kotani et al., 2001). Likewise, in NIH3T3 fibroblasts, GPR54 activation also blocked cell migration and inhibited cell proliferation through  $\text{PLC}\beta$  activation (Stafford et al., 2002). A recent



study using DNA microarray showed that KISS1 via GPR54 downregulates cell proliferation and induces apoptotic cell death in MDA-MB-435 cells through regulating cell cycle-regulating genes such as CDKN1 and GADD45 (Becker et al., 2005). Likewise, KISS1 silencing in MDA-MB-231 cells downregulated cell proliferation with a reduced ERK2 phosphorylation (Dittmer et al., 2006). However, since kisspeptin treatment or KISS1 overexpression did not affect cell proliferation in different cell types although GPR54 was endogenously expressed or exogenously overexpressed (Bilban et al., 2004; Cho et al., 2009b; Jiang et al., 2005; Masui et al., 2004; Shoji et al., 2009), a role of GPR54 signaling for cell proliferation is yet unclear.

In CHO and CHO-K1 cells, GPR54 activation phosphorylated FAK induced a formation of focal adhesion complex (Kotani et al., 2001; Ohtaki et al., 2001). In addition, GPR54 activation induced activation of RhoA downstream of FAK in renal cancer cells (Navenot et al., 2009b). Thus, those researches suggested that GPR54 blocked cell motility by inducing cell adhesion. However, RhoA activation results in the increase of cancer development and metastasis (Brantley-Sieders et al., 2008; Martin et al., 2007). RhoA activation leads to the enhanced tumor cell invasiveness via FAK-mediated amoeboid migration rather than Rac1-induced anchorage-dependent movement (Papusheva et al., 2009). Furthermore, RhoA is revealed to induce preneoplastic transformation of mammary epithelial cells (Zhao et al., 2009). As dynamics of Rho GTPases including RhoA, Cdc42, Rac1, spatiotemporally regulate cell behavior (Parsons et al., 2010; Pertz, 2010), data interpretation for roles of RhoGTPases in cancer

biology would be complex (McHenry and Vargo-Gogola, 2010). Considering those complexity, GPR54 signaling seem to be pleiotropic.

Recent studies are consistent with that notion. For example, while kisspeptin-10 induced phosphorylation of ERK and AKT in ovarian cancer cells overexpressing GPR54 (Jiang et al., 2005), kisspeptin-10 in CHO and HeLa cells overexpressing GPR54 rather blocked CXCL12-induced AKT phosphorylation but persistently enhanced ERK, resulting in apoptotic cell death (Navenot et al., 2005). KISS1-activated GPR54 transiently increased the intracellular calcium level, resulting in activation of calcium-mediated signaling pathway (Bilban et al., 2004; Navenot et al., 2005). However, it inhibited CXCL12/CXCR4-increased intracellular calcium level (Navenot et al., 2005). Although kisspeptin induced FAK phosphorylation in CHO cells (Ohtaki et al., 2001), kisspeptin inhibited VEGF-induced FAK phosphorylation in HUVEC (Cho et al., 2009b). In addition, kisspeptin-activated GPR54 in ovarian cancer cells inhibited LPA-induced PKC $\alpha$  activation (Jiang et al., 2005), while it was known to induce PKC phosphorylation and calcium mobilization (Kotani et al., 2001). GPR54 signaling increased an expression level of MCIP (also called RCAN or DSCR1) known as a calcineurin inhibitor through PKC and blocks calcineurin-dependent dephosphorylation of NFAT, resulting in suppression of NFAT-dependent transcriptional activation (Stathatos et al., 2005). Relevantly, GPR54 increased an expression of I $\kappa$ B, NF- $\kappa$ B inhibitor, and inhibited NF- $\kappa$ B-dependent MMP-9 expression (Yan et al., 2001), while it inhibited TNF $\alpha$ -dependent NF- $\kappa$ B activation by blocking RhoA (Cho et al., 2009a). Thus, GPR54 signaling might generate negative feedback system for certain intracellular

signaling pathways such as NFAT and NF- $\kappa$ B pathways. However, it is unclear whether tonic regulation of GPR54 signaling decides cellular features or GPR54 signaling is varied through different biological cues. In sum, GPR54 regulation of an intracellular signaling seems to be complex and dependent on biological cues.

Meanwhile, KISS1 silencing reduced cell aggregation of MCF7 breast cancer cells (Dittmer et al., 2008). Thus, it is plausible that GPR54 signaling may affect the initial step of tumor development, at least solid tumor formation. KISS1 expression was found to be regulated by PTHrP, TGF $\beta$  and WNT (Dittmer et al., 2006), which indicated that GPR54 function might be associated with a mechanism called EMT. Meanwhile, KISS1-activated GPR54 in hypothalamus transiently induces c-Fos expression, although it was used for readout for neuronal cell activation, which was confirmed in different species including fish (Adachi et al., 2007; Clarkson et al., 2008; Kinoshita et al., 2005). An immediate early gene, c-Fos is implicated in tumorigenesis and its gene expression is regulated by activation of RhoA, PKC, or ERK (Montaner et al., 1999; Soh et al., 1999; Wang and Prywes, 2000; Xia et al., 1996; Zhang et al., 2008). Thus, it remains to be answered that GPR54 signaling to c-Fos is unique in cancer. Likewise, GPR54 mutations revealed in physiology are yet found in cancer biology. Although homozygous mutations or compound heterozygous mutation of GPR54 showed phenotypic defects in patients, heterozygous GPR54 mutants were also able to affect normal GPR54-mediated intracellular signaling *in vitro*. Thus, it is still unclear whether heterozygous mutation of GPR54 functions dominantly in cancer while it is recessive in hypothalamic-pituitary-gonadal axis.

### 1.3 GPR54 signaling in physiology

Recent studies for GPR54 signaling have intensively focused on roles in normal physiology, especially in pubertal regulation, as GPR54 mutations were found in human patients with pubertal defects (Seminara et al., 2003). Patients carrying homozygous mutation (L148S) or compound heterozygous mutation (R331X and X399R) in GPR54 resulted in autosomal recessive idiopathic hypogonadotropic hypogonadism (IHH), which was consistent in mice and primates (de Roux et al., 2003; Elizur, 2009; Seminara et al., 2003; Wacker et al., 2008). Furthermore, a homozygous mutation of L102P also caused HH phenotype (Tenenbaum-Rakover et al., 2007). Reversely, it was found that GPR54 autosomal dominant mutation (R386P) causes idiopathic central precocious puberty (Teles et al., 2008). In addition, GPR54 polymorphism was found in promoter sequences of GPR54 gene of Chinese girls with central precocious puberty (Luan et al., 2007a), and KISS1 polymorphism for P110T with diverse haplotypes resulted in central precocious puberty in African, Chinese, and Korean women (Ko et al., 2010; Luan et al., 2007b), which indicated that mechanisms for GPR54 signaling might be important for the regulation of puberty (Navarro et al., 2004a; Navarro et al., 2004b; Nocillado et al., 2007; Richard et al., 2008). However, GPR54 deletion on chromosome 19p13.3 was not found in IHH patients (Cerrato and Seminara, 2007; Seminara et al., 2003). Mouse genetic studies were coupled GPR54 with KISS1 in sexual development (Colledge, 2009). *Gpr54* knockout mice showed a defect of reproductive system, which was relevant to clinical features in IHH patients (Colledge, 2009; Funes et al., 2003). *Kiss1* knockout mice also showed the same phenotypes but more severe than *Gpr54* knockout

mice (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007). Thus, GPR54 with KISS1 appears to be coupled in regulation of hypothalamic-pituitary-gonadal (HPG) axis. Kisspeptin-activated GPR54 induces a release of gonadotropin-releasing hormone (GnRH) for pubertal regulation (Seminara and Crowley, 2008). In detail, kisspeptin administration induced luteinizing hormone (LH) and follicle stimulating hormone (FSH) surges in rodents and primates, which was abolished by GnRH antagonist. In addition, GnRH neuron expressed GPR54 and kisspeptin administration increased c-Fos expression in GnRH neuron. Thus, GPR54 signaling was suggested to follow paracrine path in brain. GPR54, however, was expressed in hypothalamus and its localization pattern was overlapped with KISS1 localization, which indicated that GPR54 with KISS1 might evoke both autocrine and paracrine loop (Richard et al., 2009).

Recent studies further found evidence on the involvement of central GPR54 signaling in regulation of energy homeostasis, sexual development, fertility, and so on. Sexual development means that the brain develops toward male or female during perinatal period (Simerly, 2002). Although genetic factors such as sex chromosome would determine sexually dimorphic traits, sex hormones may mainly regulate sexual differentiation in brain (Simerly, 2002). GPR54 signaling appeared to control the sex hormone-regulated organization of sexually dimorphic neural populations, since *Gpr54* null mice showed female-like phenotypes (Kauffman et al., 2007b). Of note, hidden cookie test showed that female-like partner preference behavior of male *Gpr54* null mouse was not related to anosmia, suggesting that testosterone-organized sexual differentiation might be defective in *Gpr54* null mouse (Kauffman et al., 2007a).

Although it is unclear whether GPR54 signaling really works in sexual development in human, recent studies mentioned above indicate that patients carrying GPR54 mutations may show some defects in their sexual/social behaviors as well as pubertal defect. In addition, the fasting inhibits gonadotropin release and ovulation without affecting GNRH expression at hypothalamus (Bergendahl et al., 1992). KISS1 is expressed in the anteroventral periventricular nucleus (AVPV) implicated in GNRH release and in the arcuate nucleus (ARC) in energy balance (Dungan et al., 2006; Kauffman, 2010). The fasting reduced KISS1 expression level in AVPV but not ARC (Kalamatianos et al., 2008), and KISS1 expression was decreased by leptin but not insulin or insulin growth factor-1 (IGF-1) and increased by neuropeptide Y (NPY) in N6 mouse embryonic hypothalamic cell line (Luque et al., 2007). Thus, the central GPR54 signaling may be important for balancing between energy homeostasis and fertility.

Otherwise, GPR54 function for puberty is not restricted in central nervous system. Circulating kisspeptin level was higher in female than in male (Horikoshi et al., 2003). Kisspeptin was highly expressed in syncytiotrophoblasts of normal and molar pregnant placenta (Bilban et al., 2004; Janneau et al., 2002). Furthermore, in pregnant women, kisspeptin was expressed as high as 7000 fold and significantly inhibited trophoblast migration (Bilban et al., 2004). Those findings indicated that GPR54 signaling might function for pregnancy. However, patients with homozygous GPR54 mutation for L148S showed no defect in vaginal delivery of a healthy child (Pallais et al., 2006), indicating that GPR54 signaling might not be important for placental function. Thus, a direct function of GPR54 signaling in pregnancy remains yet unanswered. However, recent

studies revealed that GPR54 signaling regulated angiogenesis as well as tumor angiogenesis (Cho et al., 2009b; Mead et al., 2007), suggesting that GPR54 signaling might indirectly regulate menstruation as well as pregnancy (Dhillon et al., 2007). Furthermore, GPR54 signaling regulates angiogenesis as well as tumor angiogenesis (Cho et al., 2009b; Mead et al., 2007). GPR54 was abundant in smooth muscle of vessels including aorta, coronary artery and umbilical vein, and kisspeptins functions like vasoconstrictors in the isolated ring of coronary artery (Mead et al., 2007). Interestingly, KISS1 and GPR54 were highly detected in atherosclerotic plaque of coronary artery (Mead et al., 2007), suggesting that kisspeptins would be testable for vascular diseases. Likewise, an intratumoral injection of kisspeptin-10 inhibited tumor angiogenesis in SCID mice subcutaneously injected with PC3 prostate cancer cells through activating GPR54 expressed in human umbilical vein endothelial cells (Cho et al., 2009b). Although central KISS1 via GPR54 might regulate energy balance with food intake (Altarejos et al., 2008; Forbes et al., 2009; Kalamatianos et al., 2008), KISS1 expression was found in adipose tissue independently of hypothalamic regulation of food intake (Brown et al., 2008). Peripheral kisspeptin administration in adult male rhesus monkey increased plasma adiponectin level without affecting plasma resistin and leptin levels (Wahab et al., 2010). Furthermore, consistent with high level of KISS1 and GPR54 in pancreas, GPR54 signaling regulated insulin secretion in isolated mouse islets (Bowe et al., 2009). Altogether, recent studies focusing on functions of GPR54 signaling in metabolism strongly suggests that GPR54 signaling might have central and peripheral roles.

#### 1.4 GPR54 signaling in cancer biology

Metastasis is a critical reason for mortality in cancer patients (Joyce and Pollard, 2009). The concrete hypothesis of GPR54 function in cancer biology is its suppression of metastasis. Thus, GPR54 role in metastasis is critical for the therapeutics (Nash and Welch, 2006). However, earlier studies did not consider GPR54 existence (Lee et al., 1996; Lee and Welch, 1997). Reports published later revealed that kisspeptins were not able to function without GPR54 overexpression, although researchers used the same cell lines. In addition,  $EC_{50}$  of kisspeptin was around 10nM to 100nM in the experimental set considering GPR54 (Kotani et al., 2001; Seminara et al., 2003). However, KISS1 overexpression causes GPR54 activation with an inhibition of cell migration (Lee and Welch, 1997; Nash et al., 2007). Furthermore, KISS1 overexpression without consideration of GPR54 as a receptor could function as a suppressor of metastasis in earlier and recent studies (Lee and Welch, 1997; Nash et al., 2007). Thus, it is unclear whether KISS1 has its own inhibitory function without GPR54 activation or endogenous GPR54 requires high concentration of kisspeptins by KISS1 overexpression. Furthermore, it is still questionable whether and how GPR54 signaling only represses metastatic cancers at secondary metastatic areas (Nash and Welch, 2006).

KISS1 was first found to inhibit tumor cell migration and invasion without suppressing tumorigenesis in metastatic melanoma and breast cancer cells (Lee et al., 1996; Lee and Welch, 1997). That function of KISS1 was also studied in various cultured cell lines and cancer types (Nash and Welch, 2006). However, a role of KISS1 even in melanomas and breast cancers is still unclear, as gain-of-function studies have



limitations to identify endogenous GPR54 signaling exactly. Earlier studies using the microcell-mediated chromosome transfer of human chromosome 6 into metastatic human melanoma cell lines C8161 or MelJuSo found that DRIP-130, a cofactor required for Sp1 transcriptional activation (CRSP3) mapped at chromosome 6q16.3-q23 (~40Mb) regulated KISS1 expression (Lee and Welch, 1997; Mitchell et al., 2007; Shirasaki et al., 2001; West et al., 1998). In addition, KISS1 expression was regulated by AP2 $\alpha$  in breast cancer (Mitchell et al., 2006). Those earlier studies explain a relationship between the loss of function of KISS1 in malignant tumor and the anti-metastatic role of KISS1 gene product. Relevantly, clinical data showed KISS1 and/or GPR54 was highly and moderately increased in tumor patients (Nash and Welch, 2006). However, although GPR54 signaling seemed not to affect tumorigenesis in earlier studies and a loss of its expression was matched to cancer metastasis, recent studies showed that KISS1 was highly expressed in breast cancer as compared with normal breast tissue and cells overexpressing KISS1 induced cell attachment and invasion to matrix molecules (Martin et al., 2005). KISS1 expression was also high in uveal melanomas (Martins et al., 2008) and bladder cancer (Nicolle et al., 2007). Increased expression of KISS1 and GPR54 was further correlated to hepatocellular carcinoma (HCC) progression (Ikeguchi et al., 2003; Schmid et al., 2007), although KISS1 expression is negatively correlated with metastasis of HCC (Shengbing et al., 2009). Thus, GPR54 signaling might have certain roles prior to aggressive cancer development.

Accordingly, it was found that GPR54 signaling inhibited tumor angiogenesis, the intermediate mechanism of tumor development and metastasis (Cho et al., 2009b; Heath

and Bicknell, 2009; Ivy et al., 2009), indicating that GPR54 signaling, although GPR54 was activated by exogenous kisspeptin, could suppress tumor cell intravasation by blocking angiogenic vessel formation and inhibiting tumor cell migration. Furthermore, that study suggests that endogenous GPR54 signaling may be lost prior to tumor angiogenesis. Since hypoxia leads to tumor angiogenesis (Bertout et al., 2008; Harris, 2002), it is testable whether GPR54 signaling is involved in hypoxia.

So far, earlier studies revealed KISS1 overexpression decreased cell proliferation and soft agar colony formation (Lee et al., 1996; Lee and Welch, 1997), which indicates that KISS1 might affect tumorigenesis beyond its function against metastasis, as the anchorage-independent growth in the soft agar assays reflects primary tumor cell growth as well as metastatic tumor cell propagation. For instance, mechanisms required for primary tumor growth are similar to them for secondary metastatic tumor growth, although microenvironment at the primary organ is different from that at distant organs (Kumar and Weaver, 2009). Tumor cells need to undergo cellular mechanisms at different regions: proliferation, migration, and invasion (Kumar and Weaver, 2009). In recent studies, kisspeptins-activated GPR54 appears to regulate cell proliferation, migration, invasion, and matrix-attachment (Nash and Welch, 2006). Some studies showed data consistent with a function of GPR54 signaling for suppression of metastasis: kisspeptins-activated GPR54 mainly inhibits cell motility (Nash and Welch, 2006). However, it is still unclear whether and how GPR54 regulates cell proliferation as previous studies have found that kisspeptins-activated GPR54 signaling downregulates

cell proliferation. Thus, the nature of GPR54 function in cancer biology has to be considered in term of genetics as that in physiology has been examined.

### 1.5 GPR54 signaling in breast cancer biology

Breast cancer is one of the most frequent cancer diseases in women (Dimri et al., 2007; Henson et al., 1996; John et al., 2004). Most important steps in breast cancer progression are a conversion of ductal carcinoma in situ (DCIS) to the invasive breast carcinoma (IDC) and a distant metastasis (Dimri et al., 2007). Recent studies have defined clear relationships between genomic abnormality and histological phenotype (Sotiriou and Pusztai, 2009). The comedo subtype of DCIS possesses amplifications in HER2/Neu oncogene, which encodes the epidermal growth factor receptor (EGFR) tyrosine kinase ERBB2 (Lester et al., 2009; Rakha and Ellis, 2009). Basal-like breast cancer, an aggressive subtype of invasive ductal carcinomas with a significant aneuploidy and a lack of hormone receptor expression, are closely linked with BRCA1 mutations or with an abnormal X chromosome inactivation (Korsching et al., 2008; Lester et al., 2009; Pakkiri et al., 2009; Rakha and Ellis, 2009; Shin et al., 2009). The invasive lobular carcinoma is commonly associated with genomic losses in CDH1 encoding for E-cadherin, an important epithelial cell-cell adhesion molecule (Moriya et al., 2009).

The major population of human breast cancer is estrogen receptor (ER)-positive (Blamey et al., 2010; Kerlikowske et al., 2010; Wong et al., 2010). Especially, most low-grade ductal carcinoma in situ is ER-positive (Abdel-Fatah et al., 2008; Kerlikowske et

al., 2010), indicating that ER loss is negatively correlated with malignancy. ER loss is correlated with E-cadherin loss during metastasis (da Silva et al., 2010). However, metastatic ER-positive ( $ER^+$ ) tumor sometimes retains ER expression together with cadherin expression, indicating that  $ER^+$  tumor metastasis does not fully require dramatic changes of molecular and cellular features in  $ER^+$  non-metastatic tumor cells such as a completion of cadherin switch and/or a loss of ER (Esposito et al., 2007; Kleer et al., 2001; Pettinato et al., 2004). Furthermore, N-cadherin overexpression promotes metastasis in non-metastatic breast cancer cells where E-cadherin is abundant and N-cadherin is low (Hazan et al., 2000; Hult et al., 2007; Nieman et al., 1999). An epithelial-to-mesenchymal transition (EMT) mechanism is implicated in this switch of cancer cells (Yu et al., 2009). In breast cancer, relationships between EMT and molecular and cellular switch mechanisms of DCIS to IDC have been intensively studied. For example, an increased expression of EMT regulators such as ZEB-1, ZEB-2, SNAI-1, SNAI-2, and TWIST is correlated with metastasis even in breast cancer (Planas-Silva and Waltz, 2007; Yu et al., 2009). Along with EMT, cadherin switch is a well-known path maker for metastatic tumor progression (Hazan et al., 2004). In metastatic breast cancers, E-cadherin encoded from CDH1 gene is lost but N-cadherin from CDH2 is highly increased (Sarrio et al., 2009). Recent studies revealed a relationship of EMT with estrogen receptor. 17 $\beta$ -estradiol ( $E_2$ )-activated  $ER\alpha$  directly upregulated E-cadherin via downregulating expression of Slug, SNAI-2 gene product (Ye et al., 2008; Ye et al., 2010). Nevertheless, molecular and cellular connections between ER and EMT in breast cancer as well as normal breast are yet unclear.

Recent clinical sample studies showed that expression pattern of KISS1 and GPR54 appeared not to be limited in certain subtypes of breast cancers, although those were expressed in breast tumor cohorts (Martin et al., 2005; Pentheroudakis et al., 2010). Breast cancer brain metastasis was negatively correlated with a reduced KISS1 expression (Stark et al., 2005), which reversely indicated that KISS1 expression was high in primary breast cancer. Likewise, KISS1 expression was higher in breast tumor than in surrounding tissue (Martin et al., 2005). However, KISS1 mRNA was scarcely detected in lymph node-positive breast adenocarcinomas independently of hormone receptor status (Kostadima et al., 2007), indicating that endogenous KISS1 and GPR54 might be existing benign breast tissue and lymph node-negative breast tumor tissue. However, de Roux group showed that KISS1 and GPR54 were highly expressed in ER<sup>+</sup> breast cancer and proposed to be poor prognostic markers (Marot et al., 2007), which was not fit for recent findings that ER<sup>+</sup> cells highly expressed KISS1 (Mitchell et al., 2006). In addition, that group found that estrogen reduced KISS1 expression in MDA-MB-231 cells overexpressing ER $\alpha$  (Huijbregts and de Roux, 2010), while it increased KISS1 expression in MCF7 cells (Li et al., 2007). However, since a function of ER $\alpha$  was not completely recapitulated in MDA-MB-231 cells, highly aggressive breast cancer cells, it is still unclear whether discrepancies in recent researches missed some critical points for endogenous GPR54 signaling in breast cancer.

## 1.6 MMTV-PyMT genetically engineered mouse model systems for breast cancer research and its application to GPR54 signaling study

To the present knowledge, breast cancer is highly heterogeneous. Thus, to understand GPR54 function with view of ‘bench to bed’ or ‘bed to bench’, limitations of in vitro or in vivo studies should be considered. Mouse model cannot perfectly realize human breast cancer, although some models can reflect human breast cancer phenotype (Jonkers and Derksen, 2007; Vargo-Gogola and Rosen, 2007). Furthermore, although breast cancer subtypes are defined well, gene expression patterns of them are not perfectly matched to those of breast cancer subtypes in genetically engineered mouse model systems (Vargo-Gogola and Rosen, 2007). Cell culture system also does not show complex cellular mechanisms in heterogeneous breast tumor microenvironment, although we do not consider stromal cell components but concentrate on breast tumor (Nyberg et al., 2008; Pujana et al., 2007; Vargo-Gogola and Rosen, 2007). Thus, a first consideration is whether and how in vivo and in vitro models are fit for understanding heterogeneous breast cancer (Pujana et al., 2007; Vargo-Gogola and Rosen, 2007). In addition, those models would be relevant to human breast cancer even in terms of pathology.

Mouse models for human breast cancers are widely used in an effort to understand the biological features of breast cancer development and metastasis (Cardiff and Kenney, 2007; Degenhardt and White, 2006; Stingl and Caldas, 2007; Vargo-Gogola and Rosen, 2007). The PyMT (polyoma virus middle T antigen) mouse metastasis model was recently developed to probe the relationship between the human metastasis signature

and metastatic progression in mice, as PyMT is not expressed in human breast tumor cells whereas PyMT-induced mouse tumor model shares characteristics with human breast cancer (Lin et al., 2003; Pujana et al., 2007; Qiu et al., 2004; Theodorou et al., 2007; Vargo-Gogola and Rosen, 2007). Tumorigenesis, induced by an expression of PyMT, is under a control of mouse mammary tumor virus long terminal repeat (MMTV LTR), which results in a restricted expression of PyMT in mammary epithelia (Lin et al., 2003; Vargo-Gogola and Rosen, 2007). In PyMT mouse model system, hyperplasia is usually found as early as the onset of puberty, at approximately 3 week after birth, while aggressive carcinomas with lung metastasis are detected at 11 week (Lin et al., 2003; Vargo-Gogola and Rosen, 2007). Thus, that model system is time-saving and easy-accessing for understanding breast cancer progression including tumorigenesis and metastasis.

Loss of mammary gland development in female mice with either Gpr54 or Kiss1 knockout suggests that the Gpr54 signal has a certain role for regulation of mammary gland development (Funes et al., 2003; Lapatto et al., 2007; Seminara et al., 2003). It is plausible that no mammary gland development in Gpr54- or Kiss1-deficient mice results from the defective pubertal regulation (Funes et al., 2003; Lapatto et al., 2007; Seminara et al., 2003). However, the mouse heterozygous for Gpr54 or Kiss1 does not show any defects in pubertal regulation including postnatal mammary gland development (Funes et al., 2003; Lapatto et al., 2007; Seminara et al., 2003). In addition, GPR54 mutation did not affect lactation (Pallais et al., 2006). Otherwise, the pubertal onset initiates mammary hyperplasia in MMTV-PyMT mice (Lin et al., 2003; Vargo-Gogola and

Rosen, 2007). Together with that reason that Gpr54 heterozygosity does not affect the physiological role of Gpr54 signaling, Gpr54 heterozygous mouse crossed with MMTV-PyMT mouse is expected a good model system for understanding the endogenous GPR54 signaling in breast cancer.

### 1.7 Summary

As described, GPR54 function was first revealed in breast cancer cell lines, providing that GPR54 is a sole receptor for kisspeptins. However, there are some contradictory reports in GPR54-breast cancer studies. What make different results in literatures about GPR54 signaling? Recently, researchers considered experimental conditions when they studied GPR54 signaling in cancer biology (Marot et al., 2007). Careful considerations of experimental conditions in different studies can solve questions remained contradictory. For example, xenograft studies cannot realize tumorigenesis, as malignant tumor cells different from tumor-initiating cells are occasionally injected. Likewise, tumor is in vivo initiated from single cell but not from massive abnormal cells. Thus, those experimental limitations might fail to recapitulate innate roles of GPR54 signaling. If not, is there any missing point at our knowledge of GPR54 in cancer biology including breast cancer? Considering the earliest study, overexpressed KiSS1 suppresses metastatic melanoma and breast cancer metastasis (Lee and Welch, 1997). Later, KISS1 gene expression was revealed to be downregulated in invasive breast tumor cells (Mitchell et al., 2006). Those two results lead to the solid conclusion that KISS1 is a suppressor of cancer metastasis (Nash and Welch, 2006).



However, as mentioned above, recent clinical data concluded that breast cancers express highly increased KISS1 mRNA and moderately induced GPR54 mRNA, when compared with normal breast (Marot et al., 2007; Martin et al., 2005). In addition, chromosome 1q is amplified in breast cancer and further chromosome 6q21-32 is lost in aggressive breast cancer (Argos et al., 2008; Bilanges et al., 1999; Forozan et al., 2000; Negrini et al., 1994; Orsetti et al., 2006; Ried et al., 1995; Tsuji et al., 2010). Those genomic studies with our recent reports suggest that expression of KISS1 at chromosome 1q is increased at early stages of tumor development through a copy number increase as well as the altered expression of transcription factors such as AP2alpha and Sp1, and lost at late stages of metastasis through the loss of expression of transcriptional factors. Earlier studies focused on GPR54 signaling function in cancer metastasis on the basis of KISS1 loss in metastatic cancer and revealed a role of GPR54 signaling for the suppressing of metastasis. However, an endogenous role of GPR54 signaling in early stages of cancer development is yet evident, although clinical data show that expression of both KISS1 and GPR54 is higher in non-metastatic breast cancer than in normal breast or malignant breast cancer. Thus, missing points for GPR54 signaling may be tightly linked to experimental approaches. GPR54 studies using proper models such as genetically engineered mouse model and loss-of-function study will strengthen the understanding of GPR54 signaling in cancer biology.

Another view point following the first question is whether quantities of GPR54 or KISS1 are important for a quality control of GPR54 signaling known as a suppressor of metastasis. GPR54 tonic regulation may be linked to feedback mechanism. GPR54

signaling, as mentioned, transiently induces NF- $\kappa$ B and NFAT pathways, but prolonged GPR54 signaling inhibits those intracellular signaling through inducing their negative feedback machineries such as I $\kappa$ B and MCIP (Jiang et al., 2005; Yan et al., 2001). Those feedback mechanisms were also found in VEGF signaling that causes tonic regulation of intracellular signaling (Minami et al., 2004; Schabbauer et al., 2007). Furthermore, GPR54 signaling suppressed VEGF signaling by inhibiting FAK/Src activation, although transient activation of GPR54 and VEGF signaling independently phosphorylates FAK and Src (Abu-Ghazaleh et al., 2001; Cho et al., 2009b; Eliceiri et al., 2002; Mitra and Schlaepfer, 2006). Likewise, KISS1 inhibited TNF $\alpha$  activation of NF- $\kappa$ B by blocking RhoA activation, although KISS1 transiently induced NF- $\kappa$ B (Cho et al., 2009a; Yan et al., 2001). Thus, it is possible that high input signaling may activate another signaling pathway to control the input-driven output phenomenon. Another consideration is a possibility of GPR54 oligomerization with other membrane proteins (or by itself) as proposed earlier (Franco et al., 2007; Sakai et al., 2010; Springael et al., 2007). Recent studies found that GPR54 mutation could prolong kisspeptin-induced signaling without affecting input amplitude (Teles et al., 2008). Thus, it is testable how GPR54 signaling gains pleiotropic signaling feature.

Mouse genetics for GPR54 signaling focused on physiological roles, especially in brain. Although four *Gpr54* and two *Kiss1* transgenic mouse lines were developed and studied (Colledge, 2009), *Gpr54* signaling is yet unclear even in physiology. More importantly, there are no studies using transgenic mouse model in cancer biology. Xenograft mouse tumor model system is one of methods to elucidate gene functions and

to test functions of cancer drug. However, implanted tumor cells are different from tumor cells initiated in nature when we consider gene profiles. In addition, tumorigenesis is not developed in whole mass but propagated from single cells which are aberrantly dividing. Thus, applying transgenic mouse model is important for understanding GPR54 signaling in cancer development and metastasis.

This study investigated roles of KISS1 and GPR54 in breast cancer development and metastasis. Although earlier studies found GPR54 signaling suppressed metastasis, those missed one point that reintroduction of GPR54 signaling in metastatic cancer cells could not recapitulate non-metastatic cancer cell features completely. Correct approaches would chase endogenous GPR54 signaling from tumorigenesis to metastasis. Thus, a first study applied MMTV-PyMT mouse model with *Gpr54* knockout mouse model to examine *Gpr54* signaling during cancer progression including tumorigenesis and metastasis. Based on data, the first study concluded that GPR54 signaling was sufficient for breast tumorigenesis. A second study further asked why non-metastatic cancer cells had high level of KISS1 and GPR54. Loss-of-function study identified that knockdown of KISS1 or GPR54 altered cell morphology and induced metastasis. In sum, this study demonstrates that autocrine GPR54 signaling regulates early stages of cancer progression including tumorigenesis.

## CHAPTER II

### MATERIALS AND METHODS

#### 2.1 Cell lines, plasmids, and reagents

HEK293T, MCF7, MDA-MB-231, MDA-MB-435, T47D cells obtained from American Type Tissue Collection were maintained in DMEM with 10% FBS. MCF10A cells were grown in DMEM/F12 supplemented with 5% horse serum, 20ng/ml of EGF, 500ng/ml of hydrocortisone, 10 $\mu$ g/ml of insulin, and 100ng/ml of cholera toxin. Estradiol (E<sub>2</sub>) was purchased from Sigma-Aldrich (St. Luis, MO). Primary mammary tumors cells were isolated from mammary tumors in PyMT/GPR54<sup>+/+</sup> and PyMT/GPR54<sup>+/-</sup> female mice. In brief, to purify mammary tumor epithelial cells, tumors were minced using two scalpels until the pieces were <1mm, and then incubated in digestion buffer (0.25% trypsin-EDTA, 2mg/ml collagenase, 100U/ml hyaluronidase) for 24hr. Cells were cultured in MCF10A medium at first day, and then in DMEM medium. GPR54 siRNA was designed based on human GPR54 sequences from 330 to 360bp, and inserted into pSR-GFP/Neo. KISS1 siRNA in pSR-GFP/Neo was designed to target 170-200bp of human KISS1. GPR54-GFP was constructed by an insertion of GPR54 full length into pEGFP-N1. Carboxyl-terminally amidated kisspeptin-10 (purity>95%) was obtained from H.D Biosciences (Shanghai, China) or Genemed Synthesis (South San Francisco, CA). TNF $\alpha$  kindly received from Dr. Aggarwal B.B (UT-MDA, Houston, TX) was stocked in distilled water with 0.01% DMSO. VEGF was from R&D System provided by Biological Resources Branch, NCI-Frederick Cancer

Research and Development Center (Frederick, MD). Matrigel was from BD Biosciences (San Jose, CA).

## 2.2 In vitro studies

To examine cell growth, cells were manually counted every day until 12 days. Cell proliferation was determined using LIVE/DEAD<sup>®</sup> Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, Eugene, OR). Doubling time calculation was done using an online calculator at [www.doubling-time.com](http://www.doubling-time.com). For cell migration and invasion, scratching and Boyden chamber assays were routinely done (Cho et al., 2009b). For anchorage-independent growth, cells were cultured in 0.6% soft agar for 2 weeks. Proliferation studies were carried out using the CellTiter96 AQueous One solution cell proliferation assay. Briefly, cells plated in 96-well plate were incubated for 48-72 hours and then the AQueous One solution (Promega, Madison, MI) was added to the samples and measured at 490nm. Cells cultured in 6 well culture dishes were scratched, washed with PBS and cultured for 24hr. Cells migrated toward the wound regions were imaged and counted.  $4 \times 10^4$  cells were cultured onto matrigel-coated 8 $\mu$ m pore size chambers (BD Bioscience, San Jose, CA) and the bottom well was filled with complete medium, cells were fixed with 4% formaldehyde and stained with hematoxylin and eosin. Invaded cells were imaged and counted using Olympus IX70 invert microscope connected to digital camera DXM1200. Each experiment was performed four times, and independently done in triplicate.

### 2.3 In vivo studies

All animals were housed and maintained in a barrier facility at the Institute of Bioscience and Technology, Texas A&M System Health Science Center (Houston, TX). Transgenic FVB/N mice expressing the polyoma middle T antigen under the control of MMTV long terminal repeat promoter (MMTV-PyMT) were received from Dr. Jeffrey M. Rosen and Dr. Jianming Xu at Baylor College of Medicine (Houston, TX). All matings were performed with male mice heterozygous for the PyMT transgene and female C57BL/6 GPR54<sup>+/-</sup> mice. First, PyMT/GPR54<sup>+/+</sup> and PyMT/GPR54<sup>+/-</sup> mice were generated by crossing PyMT male mice with GPR54<sup>+/-</sup> female mice. Then, PyMT/GPR54<sup>+/-</sup> male mice were interbred with GPR54<sup>+/-</sup> female mice, yielding PyMT/GPR54<sup>+/+</sup>, PyMT/GPR54<sup>+/-</sup>, and PyMT/GPR54<sup>-/-</sup> mice (Figure 2.1). Offspring were genotyped by polymerase chain reaction of genomic DNA from tail clippings (Figure 2.1). Genotyping for the PyMT transgene was performed according to the protocol of Jackson Laboratories (Bar Harbor, ME). All mice analyzed in this study were virgin females. Animal protocols used for this study were approved by the Institute of Bioscience and Technology for Animal Studies.

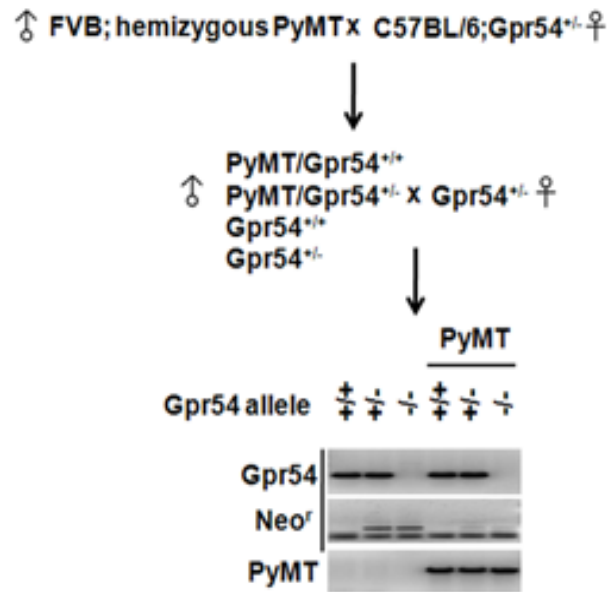


Figure 2.1 PyMT/Gpr54 mouse generation and genotyping.

Pubertal phenotypes such as vaginal opening at a precise time schedule showed no differences between PyMT/GPR54<sup>+/+</sup> and PyMT/GPR54<sup>+/-</sup> mice, which is consistent with previous reports that Gpr54 (as well as Kiss1) heterozygous condition does not significantly alter a level of sexual hormones and not affect pubertal development (Funes et al., 2003; Lapatto et al., 2007; Seminara et al., 2003). Lack of tumor development in PyMT/Gpr54<sup>-/-</sup> mice indicates that hypothalamic Gpr54 tightly regulates postnatal mammary development, which is consistent with previous findings that Gpr54 knockout mice do not have postnatal mammary gland development and that PyMT-induced tumors develop following pubertal mammary gland development (Funes et al., 2003; Lapatto et al., 2007; Lin et al., 2003; Seminara et al., 2003). Ovariectomy experiments where heterozygous and null mouse were ovariectomized and pelleted with E<sub>2</sub> support that mammary Gpr54 might not locally function for postnatal mammary gland development

(Figure 2.2). Altogether, those data indicate that Gpr54 heterozygosity results in the haploinsufficiency for PyMT-induced mammary tumor development, which is not due to the pubertal defect of mammary gland development.

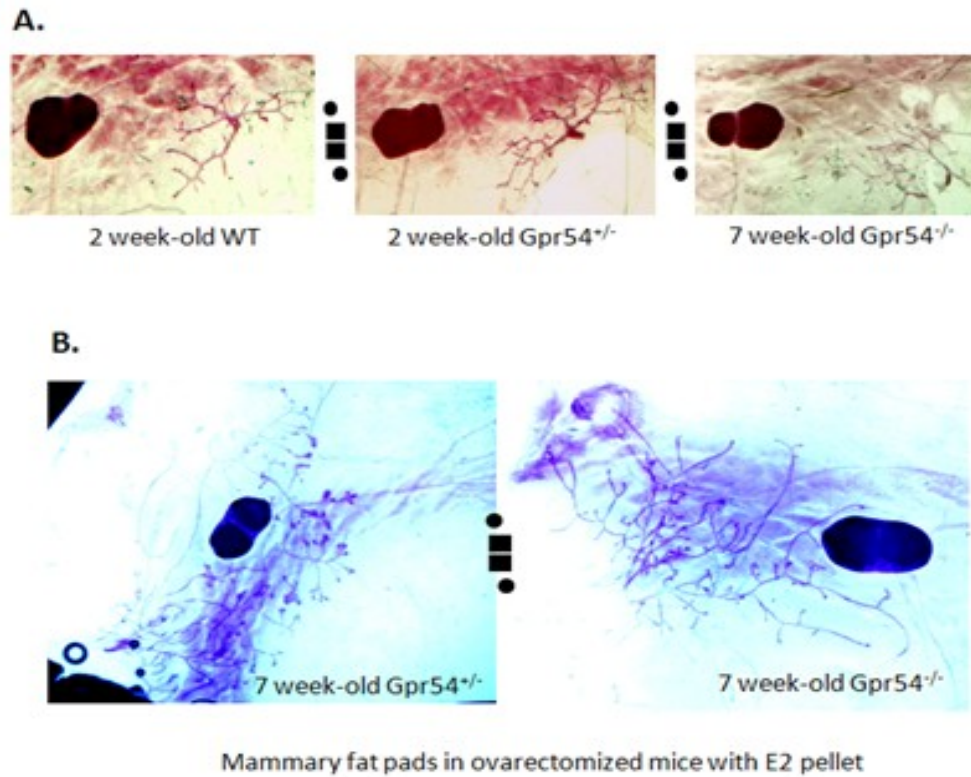


Figure 2.2 Gpr54 signaling does not work for postnatal mammary development. (A) Mammary glands in 2 week-old wild type (WT) and Gpr54 heterozygous (GPR54<sup>+/-</sup>) and 7 week-old Gpr54 null (GPR54<sup>-/-</sup>) mice. (B) Mammary gland development in ovariectomized Gpr54 heterozygous and null mice.

Mice were observed three times a week for mammary tumors by eye examination and finger palpation. Tumor length (L) and width (W) were measured once a week by a caliper and tumor volume was estimated using the standard calculation for a sphere  $\frac{4}{3} \times$



$(L \times W^2) \times \pi$ . Mice were euthanized at different stages of mammary tumorigenesis, and their mammary glands and tumors were collected for morphologic and biochemical analyses. For the whole-mount staining, the fourth inguinal mammary fat pads were excised, fixed with ethanol, and then stained with carmine alum. For the histological study, tissues were formalin fixed for 24hrs, and embedded in paraffin. 5 $\mu$ m in width were cut, stained with hematoxylin and eosin, and evaluated by 3 different observers. Analyses and descriptions were performed in accordance with the guidelines in the NIH mammary gland pathology website (<http://mammary.nih.gov/index.html>).

1x10<sup>6</sup> primary tumor cells isolated from MMTV-PyMT mouse were mixed with 0.1% matrigel and then injected into the left 4<sup>th</sup> mammary fat pad of NOD.SCID/NCr mouse (NCI-Frederick, Frederick, MD). Primary tumor growth was examined over 24 days. Human breast tumor cells (1x10<sup>6</sup> per mouse) were mixed with 0.1% matrigel (BD Bioscience, Bedford, MA) and then injected into the left 4<sup>th</sup> mammary fat pad of 6week-old virgin female Nu/Nu mouse (NCI-Frederick, Frederick, MD). Tumor growth was examined over 24 days. Estradiol (E2) pellets (1.7mg per pellet, 60 days release; innovative Research of American, Sarasota, FL) were subcutaneously implanted. Kisspeptin (1 $\mu$ M) was twice a week until the end of experiments. Tumor length and width were measured twice a week using a caliper and tumor volume was estimated using the standard formula  $0.52 \times (\text{length} \times \text{width}^2)$ . For experimental metastasis assays, tumor cells (3x10<sup>6</sup> per mouse) were injected i.v. into Nu/Nu mouse. 20 days after injection, pulmonary metastatic foci number was counted and lung section was done.

## 2.4 Immunohistochemistry

Paraffin-embedded tissues were sectioned at 5 $\mu$ m. Sections were then deparaffinized first by treatment with xylene for 3min two times and rehydrated by passage through a graded series of ethanol. Antigen retrieval was performed by boiling the slides in 10mM sodium citrate buffer, pH 6.0 for 20min. Endogenous peroxidase activity was quenched by incubating the slides for 1min in 3% H<sub>2</sub>O<sub>2</sub> diluted in methanol. Slides were then washed in phosphate-buffered saline (PBS) plus 1% Tween 20 and blocked with a solution containing 3% BSA, 0.1% Triton X-100 in PBS for 1hr at room temperature. Samples were washed three times with PBS for 10 min each. Next procedures were done according to the manufacturers' manuals. HistoStain<sup>®</sup> Plus Broad Spectrum (DAB) (Invitrogen, Camarillo, CA) or Vectorstain ABC kit with Vector<sup>®</sup> NovaRED<sup>™</sup> Substrate kit (Vector Laboratories, Burlingame, CA) were used. Anti-Gpr54, -KISS1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted with 1:100 and incubated overnight at 4°C. For human GPR54, anti-GPR54 antibody obtained from Abcam (Cambridge, MA) was used in 1:500 dilutions.

## 2.5 RT-PCR, real time quantitative PCR, luciferase assay, EMSA, and ChIP assay

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). RT-PCR was done with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MTV RT) (Promega, Madison, WI). The real time PCR was performed using RT<sup>2</sup> Real-Time<sup>™</sup> SYBR Green/ROX PCR master mix (SABiosciences, Frederick, MD) in ABI PRISM<sup>®</sup> 7900 HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA).

## 2.6 Immunoblotting

Protein concentration was determined using BCA kit (Pierce, Rockford, IL) and the volume required for 50 $\mu$ g of protein was decided. Samples were then separated by 6-15% SDS-PAGE and transferred to PVDF membrane. The membrane was stained with Ponceau S, washed, and then followed by the immunoblot analysis. All subsequent wash buffers contained 10mM Tris pH8.0, 150mM NaCl, 0.05% Tween-20, which was supplemented with 1% bovine serum albumin (BSA) and 2% non-fat dry milk for blocking solution and 1% BSA for the antibody diluent. Primary antibodies were used at 1:500 dilutions. Horseradish peroxidase-conjugated secondary antibodies were diluted at 1:10,000. Anti-GPR54, RhoA, Cdc42, Rac1, and Ras antibodies were used (Santa Cruz Biotechnology, Santa Cruz, CA). 50 $\mu$ g of protein from cells lysed with RIPA buffer was loaded onto 8-12% SDS-gel, transferred to PVDF membrane, and incubated with the appropriate antibodies. Phosphorylated form of c-Src was detected using anti-c-Src pY416 antibody (Cell Signaling, Danvers, MA). To examine FAK phosphorylation, anti-FAK Y397 and anti-FAK Y576/577 (Cell Signaling, Danvers, MA) were used. Antibodies for KISS1, GPR54, JNK, pJNK, pERK, ERK, pp38, p38, ERalpha, Sp1, and VEGF were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibodies for N-cadherin, E-cadherin, P-cadherin, Pan-cadherin, ZEB1, Snail, Slug, and Actin were purchased from Cell Signaling Technology (Danvers, MA). For the Rho GTPases activity, substrates such as GST-PAK PBD or GST-Rhotekin RBD induced in *E. coli* were pulled down, incubated with GST-Sepharose beads, and then mixed with 500 $\mu$ g whole protein. To detect active Rho GTPases, the appropriate antibody such as anti-

RhoA (Santa Cruz Biotech, Santa Cruz, CA), anti-Cdc42 (Santa Cruz Biotech, Santa Cruz, CA), or anti-Rac1 (Santa Cruz Biotech, Santa Cruz, CA) was used. Substrate-bound Rho GTPases present their active state. Protein concentration was determined using BCA kit (Pierce, Rockford, IL). Anti-GPR54 and anti-KISS1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies were obtained from Cell Signal (Cell Signaling Technology, Danvers, MA).

## 2.7 Statistics

Statistics were done using MedCalc (Medcalc Software, Mariakerke, Belgium). All statistics are two-sided. One-way analysis of variance and student's t test were appropriately done. Tumor-free survival curves were performed by Kaplan-Meier method and compared with log-rank test. Some data were expressed as means with 95% confidential intervals. A P value  $< .05$  was considered to indicate statistical significance.

## CHAPTER III

### RESULTS

#### 3.1 Autocrine GPR54 signaling is required for tumor promotion in dosage-dependent manner

##### 3.1.1 Gpr54 heterozygosity delays breast tumor development

To investigate breast tumor development, palpable tumors were observed until 16 weeks. When palpable tumors in PyMT/Gpr54<sup>+/+</sup> ( $n=27$ ), PyMT/Gpr54<sup>+/-</sup> ( $n=33$ ), and PyMT/Gpr54<sup>-/-</sup> mice ( $n=15$ ) was examined using Kaplan-Meier analysis, PyMT/Gpr54<sup>+/-</sup> mice showed a delay of breast tumor development in comparison with PyMT/Gpr54<sup>+/+</sup> mice, whereas PyMT/Gpr54<sup>-/-</sup> mice generated no palpable tumor (Figure 3.1A). 50% of PyMT/Gpr54<sup>+/+</sup> mice developed palpable tumors in mammary fat pads at 7 weeks of age, and 50% of PyMT/Gpr54<sup>+/-</sup> mice did at 10 weeks of age (Figure 3.1A;  $p=0.0012$ , PyMT/Gpr54<sup>+/+</sup> mice vs. PyMT/Gpr54<sup>+/-</sup>), indicating Gpr54 heterozygosity affects tumor latency. Next, we counted breast tumor number in different mammary fat pads of each mouse whole body at 11 weeks. PyMT/Gpr54<sup>+/+</sup> ( $n=7$ ) and PyMT/Gpr54<sup>+/-</sup> mice ( $n=7$ ) had 5 to 15 and 0 to 7 tumors, respectively (Figure 3.1B and 3.1C;  $p=0.0001$ , PyMT/Gpr54<sup>+/+</sup> vs. PyMT/Gpr54<sup>+/-</sup>). Thus, Gpr54 heterozygosity further affects breast tumor multiplicity. We then measured tumor burden at 15 week in order to analyze whether the delay of tumor latency under Gpr54 heterozygosity affects tumor growth. Tumor volume at 15 week was smaller in PyMT/Gpr54<sup>+/-</sup> mice ( $n=8$ ) than in

PyMT/Gpr54<sup>+/+</sup> mice ( $n=8$ ) (Figure 3.1D and 3.1E;  $p=0.001$ , PyMT/Gpr54<sup>+/+</sup> vs. PyMT/Gpr54<sup>+/-</sup>). To summarize, our data indicate that Gpr54 delays breast tumor latency and multiplicity, and further affects tumor growth.

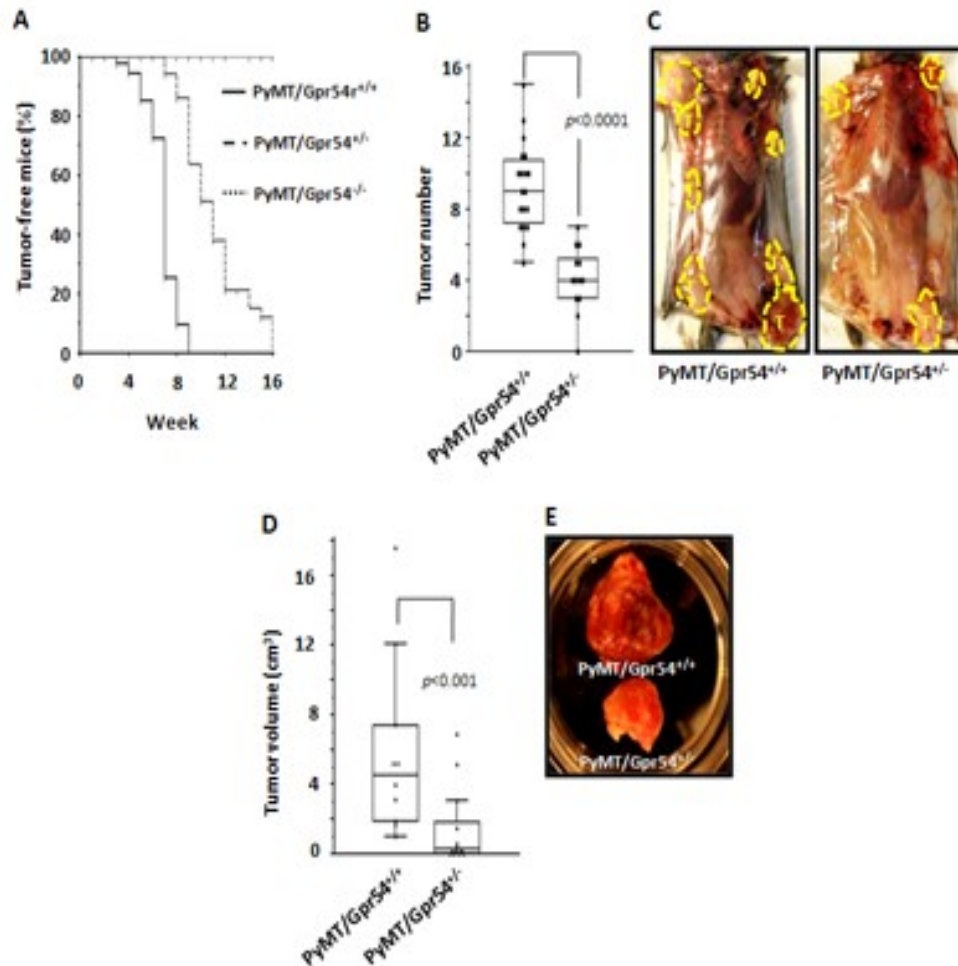


Figure 3.1 Gpr54 heterozygosity affects breast tumor latency, multiplicity, and growth. (A) Kaplan-Meier plot for palpable tumors examined until 16 weeks. (B) Breast tumor numbers in different mammary fat pads. (C) Mice sacrificed at 11 week. T indicates

tumor. (D) The volume of primary tumors in 11 week-old mice. (E) Representative tumor images.

### 3.1.2 Gpr54 heterozygosity delays lung metastasis

PyMT-induced breast tumors primarily metastasize to lung, which is normally detected at 11 weeks of age (Lin et al., 2003; Vargo-Gogola and Rosen, 2007). To investigate whether Gpr54 heterozygosity affects metastatic tumor growth at lung, we prepared lung sections and measured metastatic tumor areas. When lung in 11 week-old PyMT/Gpr54<sup>+/+</sup> or PyMT/Gpr54<sup>+/-</sup> mice was sectioned, metastasized tumor area in PyMT/Gpr54<sup>+/-</sup> lung was much smaller than that in PyMT/Gpr54<sup>+/+</sup> mice (Figure 3.2A and 3.2B). Metastatic tumor areas reached an average 1mm<sup>2</sup> in PyMT/Gpr54<sup>+/+</sup> lung, whereas those did not reach 0.5mm<sup>2</sup> in PyMT/Gpr54<sup>+/-</sup> lung (Figure 3.2A;  $p=0.0007$ , PyMT/Gpr54<sup>+/+</sup> vs. PyMT/Gpr54<sup>+/-</sup>). Thus, our data indicate that Gpr54 heterozygosity affects metastatic tumor growth at lung.

To investigate whether Gpr54 heterozygosity affects a rate of metastatic tumor foci along with metastatic tumor growth, we counted lung metastatic tumor foci in PyMT/Gpr54<sup>+/+</sup> ( $n=5$ ) and PyMT/Gpr54<sup>+/-</sup> mice ( $n=5$ ). When we examined metastasized tumor foci at 15 week, the foci number found in PyMT/Gpr54<sup>+/+</sup> lung was higher than that in PyMT/Gpr54<sup>+/-</sup> lung (Figure 3.2C and 3.2D;  $p=0.04$ , PyMT/Gpr54<sup>+/+</sup> vs. PyMT/Gpr54<sup>+/-</sup>). Thus, our data indicate that the delay of breast cancer development by Gpr54 haploinsufficiency further slows lung metastasis down.

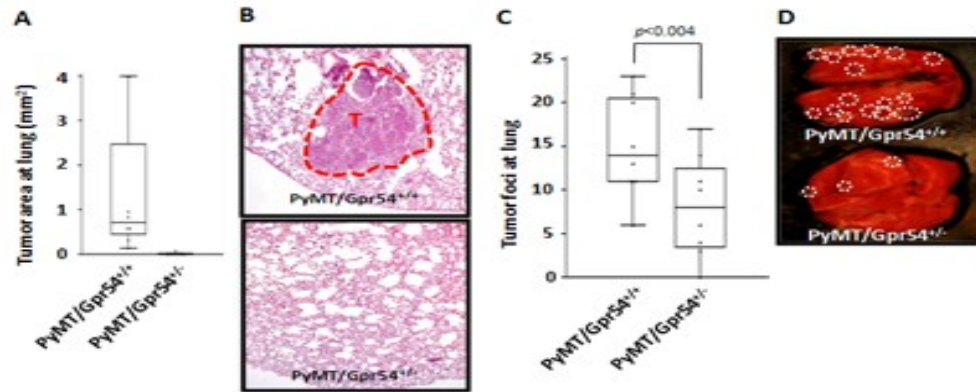


Figure 3.2 Gpr54 heterozygosity delays lung metastasis. (A) Metastatic tumor area in lung at 11 week (B) Lung tissues stained with hematoxylin and eosin. T indicates metastatic tumor area. (C) Macroscopic tumor foci number on lung surface at 15 week. (D) Representative lung images. Circles indicate metastatic tumors.

### 3.1.3 Gpr54 heterozygosity delays the incidence of breast hyperplasia and tumor formation

As Gpr54 heterozygosity affected tumor latency, multiplicity, and growth, we next examined whether these results are due to a delay of tumor incidence. To analyze mammary tumor incidence, fourth inguinal mammary fat pads at the age of 5 to 9 weeks were whole-mounted, since whole mounting preparations are preferred to address premalignant lesions of mammary glands, termed mammary intraepithelial neoplasia (MIN) or hyperplastic atypia that result in mammary tumors (4-6). When hyperplastic nodules in the 4<sup>th</sup> inguinal mammary fat pad of PyMT/Gpr54<sup>+/+</sup> ( $n=20$ ), PyMT/Gpr54<sup>+/-</sup> ( $n=21$ ), and PyMT/Gpr54<sup>-/-</sup> ( $n=7$ ) mice at 5 to 9 week were counted, total number of hyperplastic nodules was less in the 4<sup>th</sup> inguinal mammary fat pad of PyMT/Gpr54<sup>+/-</sup>



mice than in that of PyMT/Gpr54<sup>+/+</sup> mice (Figure 3.3A and 3.1.3B;  $p=0.001$ , PyMT/Gpr54<sup>+/+</sup> vs. PyMT/Gpr54<sup>+/-</sup>). In addition, PyMT/Gpr54<sup>-/-</sup> did not develop hyperplastic mammary glands (Figure 3.3B), as the absence of central Gpr54 function was linked to defective postnatal mammary gland development. At 5 week, mammary ductal hyperplasia in PyMT/Gpr54<sup>+/-</sup> mice was less severe than that in PyMT/Gpr54<sup>+/+</sup> mice (Figure 3.3B, inboxes in second panels). At 7 to 9 week, tumor mass was smaller in PyMT/Gpr54<sup>+/-</sup> mice than in PyMT/Gpr54<sup>+/+</sup> mice (Fig. 3.3A, arrows in third and forth panels). Thus, our data indicate that Gpr54 heterozygosity affects tumor incidence and formation.

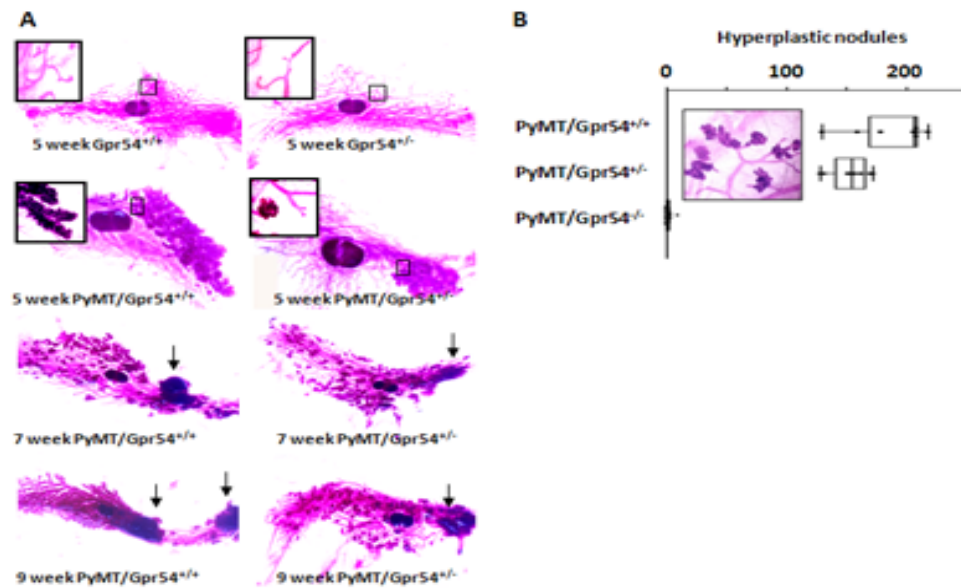


Figure 3.3 Gpr54 heterozygosity attenuates the incidence of mammary hyperplasia, solid tumor formation, and malignance. (A) Primary tumor development in 4<sup>th</sup> inguinal mammary fat pad was examined at 5 to 9 week of age. Arrows indicate primary tumors. (B) Hyperplastic nodules (the inbox) at 5 week were counted and graphed. Inbox represents the hyperplastic nodule image.

### 3.1.4 Gpr54 heterozygosity affects malignancy

To understand a delay of mammary cancer progression in PyMT/Gpr54<sup>+/-</sup> mice, we analyzed different types of breast cancers in 11 week-old PyMT/Gpr54<sup>+/+</sup> ( $n=6$ ) or PyMT/Gpr54<sup>+/-</sup> ( $n=6$ ) mice. Tumor cohorts were more complex and advanced in histology samples of PyMT/Gpr54<sup>+/+</sup> mice than in those of PyMT/Gpr54<sup>+/-</sup> mice (Figure 3.4). Thus, to investigate whether Gpr54 heterozygosity further affects tumor malignancy, we analyzed different tumor types in tumor cohorts of PyMT/Gpr54<sup>+/+</sup> and PyMT/Gpr54<sup>+/-</sup> mice according to the histology definition in the NIH mammary gland pathology website (<http://mammary.nih.gov/index.html>). Normal mammary gland (Terminal ductal lobular unit: TDLU) was not found in PyMT/Gpr54<sup>+/+</sup> mice, but approximately 25% of TDLU was detected in PyMT/Gpr54<sup>+/-</sup> mice (Figure 3.4). Likewise, Gpr54 heterozygosity markedly delayed mammary tumor progression with a decrease of DCIS by an approximate 20%, when tumor cohorts in PyMT/Gpr54<sup>+/-</sup> mice were compared with those in PyMT/Gpr54<sup>+/+</sup> mice (Figure 3.4). Moreover, the amount of IDC in PyMT/Gpr54<sup>+/-</sup> mice was lower than those in PyMT/Gpr54<sup>+/+</sup> mice, amounting to an approximate 30% in 11 week-old PyMT/GPR54<sup>+/+</sup> mice compared to an approximate 15% in 11 week-old PyMT/Gpr54<sup>+/-</sup> mice (Figure 3.4).

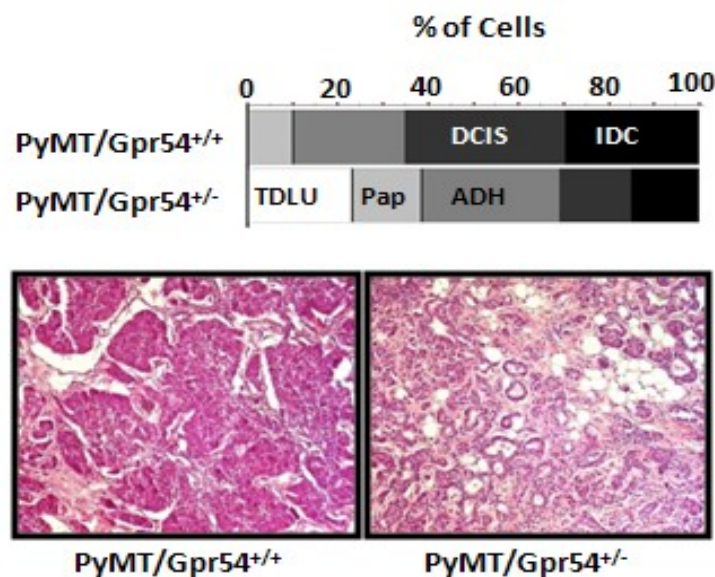


Figure 3.4 Gpr54 heterozygosity attenuates malignancy. Tumor malignancy was presented by observing different types of cancers in cancer cohorts. Hematoxylin and eosin staining of 11week-tumor tissues (bottom). Terminal ductal lobular unit: TDLU; Papillomas: Pap; Atypical ductal hyperplasia: ADH; Ductal carcinoma in situ: DCIS; Invasive ductal carcinoma: IDC.

### 3.1.5 Gpr54 expression pattern in tumor progression

Next, we examined the *in situ* localization of Gpr54. When we performed the immunohistochemistry with anti-Gpr54 antibody, Gpr54 was mainly expressed in mammary tumors (Figure 3.5). Importantly, Gpr54 expression was higher in well-differentiated tumors but decreased in poorly differentiated tumors (Figure 3.5). In addition, Gpr54 was expressed in lung metastatic tumor (Figure 3.5). Thus, Gpr54 in non-malignant tumor might function for tumor development at early stages.

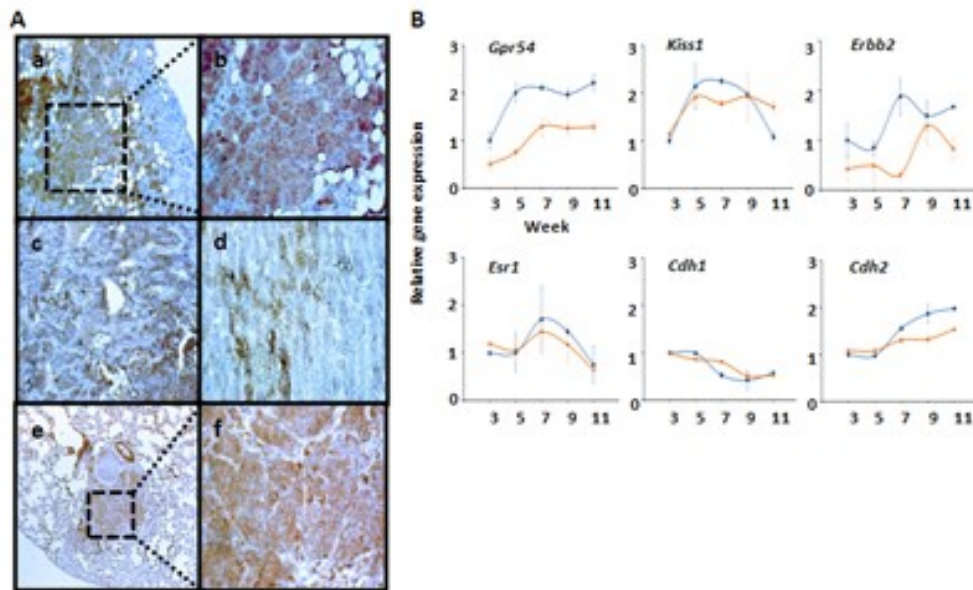


Figure 3.5 Gpr54 expression in breast cancer (D) Gpr54 immunohistochemistry in tumor cohorts. (D-a) Gpr54-stained 15 week of PyMT/Gpr54<sup>+/-</sup> tumor. Magnification was 20x. (D-b) Enlarged tumor area expressing Gpr54 in a box of (D-a). Magnification, 40x. (D-c) 11 week of PyMT/Gpr54<sup>+/+</sup> tumor section stained with anti-Gpr54 antibody. (D-d) 15 week of PyMT/Gpr54<sup>+/+</sup> tumor mass stained with anti-Gpr54 antibody. (D-e) 11 week of PyMT/Gpr54<sup>+/+</sup> lung stained with anti-Gpr54 antibody. (D-f) A box region in (D-e) was enlarged with 40x magnification. (E) Gene expression profiles during mammary tumor progression.

The immunohistochemistry data showed that Gpr54 expression in breast cancer was changed during cancer progression. Thus, an expression pattern of breast epithelial Gpr54 in different time points was investigated. In brief, RNA from mammary glands with tumors at each time point, and performed real time PCR with primers for *Gpr54*, *Kiss1*, *ErbB2*, *ERα*, *E-Cadherin*, and *N-Cadherin*. *Gpr54* expression level was increased

when hyperplasia was detected, and Gpr54 heterozygosity affected the decrease of the *Gpr54* expression level (Figure 3.5A, 5B, and 5G). Considering Gpr54 staining pattern in tumor cohorts (Figure 3.5A, 5C, and 5D), Gpr54 expression might be regulated translationally or post-translationally. Consistent previous studies, *Kiss1* expression level was high during tumor progression and decreased at metastatic stage, and Gpr54 heterozygosity appeared to affect its expression when tumors metastasized. Gpr54 heterozygosity affected *ErbB2* expression during tumor development, as an increase of *ErbB2* expression was detected at 7 week in PyMT/Gpr54<sup>+/+</sup> and at 9 week in PyMT/Gpr54<sup>+/-</sup> (Fig. 3.5G). However, both *ERα* and *E-Cadherin* did not show any significantly different expression pattern between PyMT/Gpr54<sup>+/+</sup> and PyMT/Gpr54<sup>+/-</sup>. Especially, *N-Cadherin* representing tumor cell invasiveness was affected by Gpr54 heterozygosity, which was reversely correlated with *Kiss1* expression pattern. In epitome, mammary epithelial Gpr54 heterozygosity seems to affect tumoral Gpr54 expression and further the expression of genes relating tumor cell invasiveness.

### 3.1.6 Gpr54 heterozygosity in breast tumor cells affects tumorigeneity in vitro

In order to understand a function of Gpr54 signaling in mammary tumors, mammary tumor cells were isolated from tumor burdens in 11 week-old PyMT/Gpr54<sup>+/+</sup> and PyMT/Gpr54<sup>+/-</sup> mice. PyMT/Gpr54<sup>+/-</sup> cells compared to PyMT/Gpr54<sup>+/+</sup> cells expressed less Gpr54 mRNA, while *Kiss1* mRNA level was not altered in both cells (Figure 3.6A). PyMT/Gpr54<sup>+/+</sup> and PyMT/Gpr54<sup>+/-</sup> primary tumor cells were cultured up to 12 days and cell number was counted every day to calculate the doubling time. Doubling time of

PyMT/Gpr54<sup>+/+</sup> and PyMT/Gpr54<sup>+/-</sup> primary tumor cells was 29.33hr and 31.76hr, respectively (Figure 3.6B;  $p=0.0018$ , PyMT/Gpr54<sup>+/+</sup> vs. PyMT/Gpr54<sup>+/-</sup>). Thus, data suggest that mammary Gpr54 signaling appears to be required for mammary tumor cell proliferation. Cell migration of PyMT/Gpr54<sup>+/+</sup> and PyMT/Gpr54<sup>+/-</sup> primary tumor cells was next examined. Cells were cultured on Boyden chamber precoated with matrigels and then migrated cell number was counted after 24hr. The migration capacity of PyMT/Gpr54<sup>+/+</sup> primary tumor cells (average=130) was higher than that of PyMT/Gpr54<sup>+/-</sup> primary tumor cells (average=85) (Figure 3.6C;  $p=0.0104$ , PyMT/Gpr54<sup>+/+</sup> vs. PyMT/Gpr54<sup>+/-</sup>). Next, to examine if Gpr54 heterozygosity in mammary tumor cells affects tumorigenesis, the anchorage-independent growth assay were done. PyMT/Gpr54<sup>+/+</sup> and PyMT/Gpr54<sup>+/-</sup> primary tumor cells were cultured in soft agar for 20 days and then colonies were counted. Compared to PyMT/Gpr54<sup>+/+</sup> cells, PyMT/Gpr54<sup>+/-</sup> cells formed colonies less in number (Figure 3.6D;  $p=0.033$ , PyMT/Gpr54<sup>+/+</sup> vs. PyMT/Gpr54<sup>+/-</sup>). Thus, Gpr54 in mouse breast epithelial cells might affect tumorigeneity.

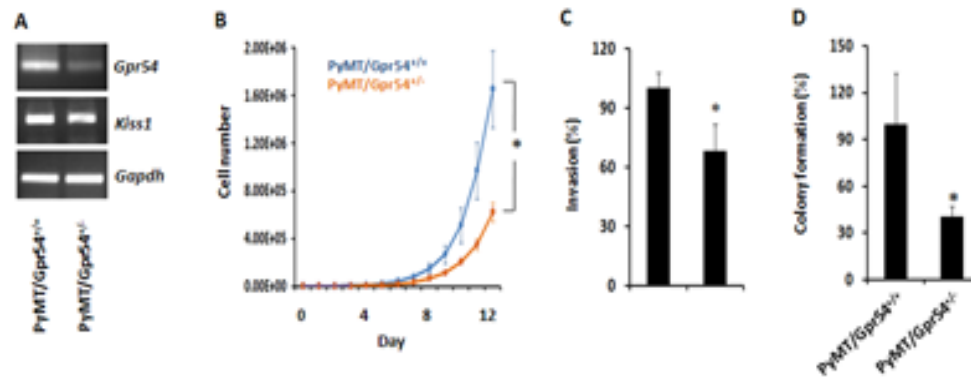


Figure 3.6 Gpr54 signaling regulates tumorigenesis. (A) *Gpr54* and *Kiss1* mRNA level in primary tumor cells. *Gapdh* for the internal control. (B) Primary tumor cell growth. (C) Cell invasiveness. (D) Anchorage-independent growth.

### 3.1.7 Gpr54 heterozygosity in breast tumor cells affects tumorigenesis in vivo

In order to test the assumption that mammary epithelial Gpr54 heterozygosity affected tumorigenesis, the orthotopic injection assays were done, since Gpr54 genotype is only different in tumors injected. Two tumor cell lines were injected into the 4<sup>th</sup> mammary fat pads of NOD.SCID/NCr mice (n=7 for each tumor cell line), and then tumor growth was observed for 4 weeks. *PyMT/Gpr54<sup>+/+</sup>* tumor cells grow faster than *PyMT/Gpr54<sup>+/-</sup>* tumor cells (Figure 3.7).

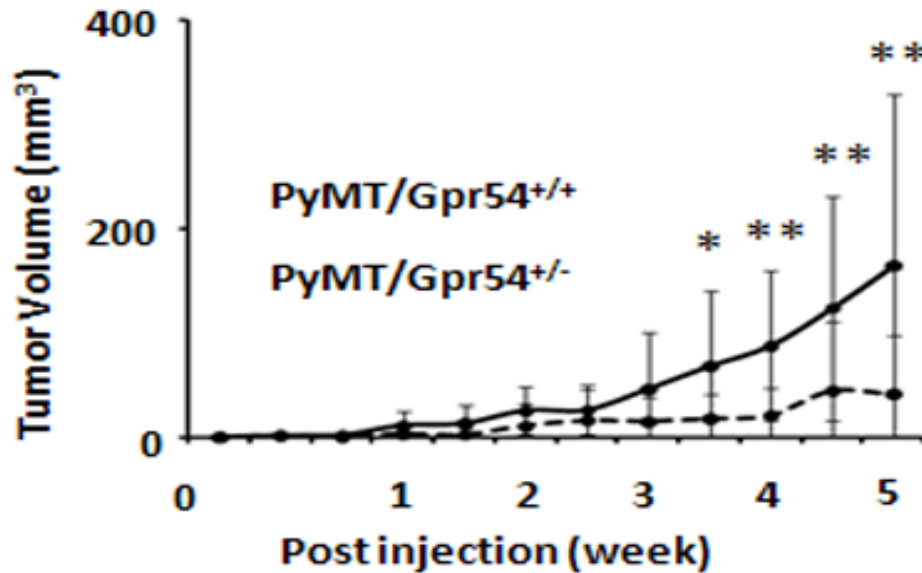


Figure 3.7 Gpr54 heterozygosity attenuates tumorigenicity in vivo. The orthotopic tumor growth. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ .

### 3.1.8 Gpr54 activation of RhoA supports Ras-induced tumorigenesis

In order to understand Gpr54 signaling for tumorigenesis, we next examined an activity of Rho GTPases that is known to be regulated by GPR54 (Cho et al., 2009a; Navenot et al., 2009a). In PyMT/Gpr54<sup>+/+</sup> tumor cells, Gpr54 knockdown significantly decreased RhoA activity but did not affect an activity of either Cdc42 or Rac1. RhoA activity in PyMT/Gpr54<sup>+/-</sup> tumor cells was also decreased when compared to that in PyMT/Gpr54<sup>+/+</sup> tumor cells (Fig. 3.8A). Thus, these data indicate that Gpr54 regulates RhoA activity basally in mammary tumor cells.

To link this finding with our previous observation that Gpr54 affects tumorigenesis, we next performed the colony formation assays. Dominant negative RhoA (RhoA N17) decreased the colony formation of PyMT/Gpr54<sup>+/+</sup> tumor cells, but wild type and



constitutively active RhoA (RhoA V14) increased the colony formation. These phenomena were also replicated in PyMT/Gpr54<sup>+/-</sup> tumor cells even though that colony number was less than wild type tumor cells (Figure 3.8B). Altogether, these data indicate that RhoA downstream of Gpr54 functions for mammary tumorigenesis.

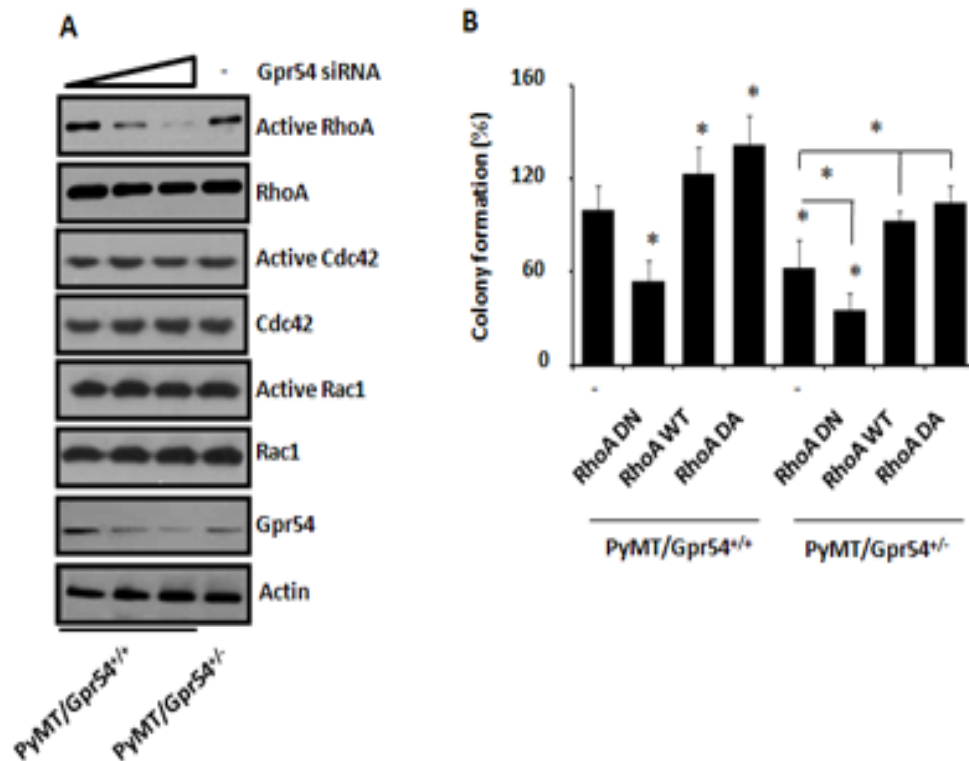


Figure 3.8 Gpr54 signaling via RhoA regulates tumorigenesis. (A) Gpr54 dosage-dependent regulation of RhoA. (B) Anchorage-independent growth. \*,  $p < 0.05$ .

### 3.1.9 Expression patterns of human KISS1 and GPR54 in the normal breast tissue and different types of breast cancer

We next examined an expression pattern of human KISS1 and GPR54 in the normal breast tissue and different types of cancers, since our data showed that the expression of both KISS1 and GPR54 was altered during cancer progression. While KISS1 and GPR54 were expressed in normal breast tissue, hyperplasia, fibroadenoma, ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), the immunoreactive intensity of both proteins was higher in hyperplasia, fibroadenoma and ILC than in normal tissue, DCIS and IDC (Figure 3.1.9). Those data from human tissue samples were consistent with data from the mouse PyMT-induced tumor tissue samples, suggesting that autocrine GPR54 signaling has certain roles at early stages of breast cancer development.

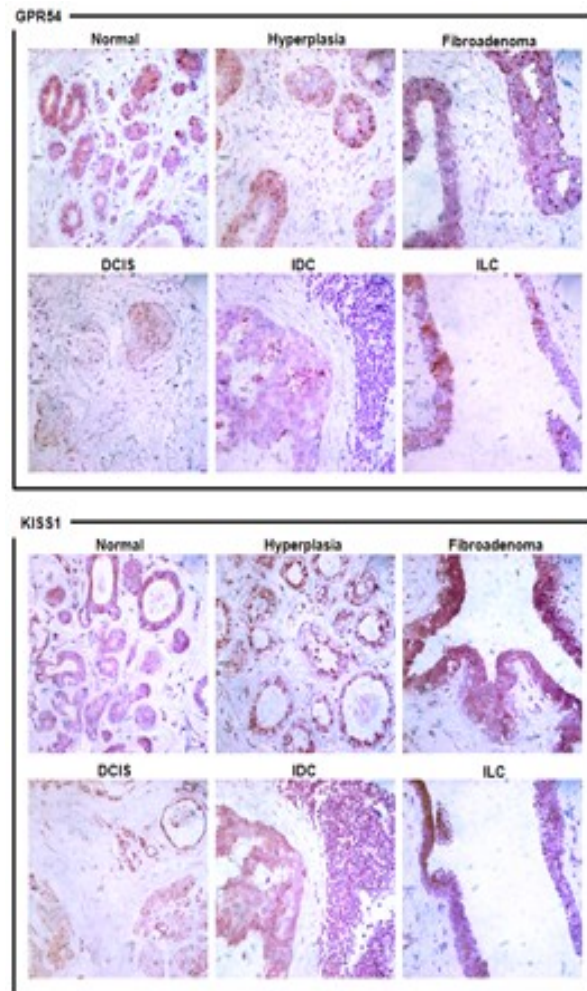


Figure 3.9 Expression pattern of KISS1 and GPR54. The immunohistochemistry for KISS1 and GPR54 was performed using human tissue micro array platform. Magnification, 40x.

### 3.1.10 Autocrine GPR54 signaling in normal breast epithelial cells sufficiently regulates Ras-induced breast tumorigenesis through RhoA

Next, MCF10A normal breast epithelial cells were overexpressed with constitutively active Ras (Ras V12) to induce Ras-mediated tumorigenesis, as PyMT-induced tumorigenesis follows an oncogenic activation of Ras (Pylayeva et al., 2009; Rodriguez-Viciano et al., 2006). MCF10A overexpressing constitutively active Ras showed that GPR54 knockdown decreased RhoA activation (Figure 3.10A). Next, when it was examined if GPR54 affects Ras-induced MCF10A colony formation, GPR54 knockdown using GPR54 siRNA decreased Ras-induced colony formation of MCF10A (Figure 3.10B), indicating that GPR54 signaling is involved in Ras-induced oncogenesis. Colony formation assays were further performed in order to examine if GPR54-mediated RhoA activation functions in Ras-induced tumorigenesis. As shown in figure 3.10C, dominant negative RhoA decreased Ras-induced colony formation of MCF10A. Furthermore, constitutively active RhoA recovered GPR54 defect in Ras-induced tumorigenesis, indicating that RhoA is downstream of GPR54 in tumorigenesis. In addition, RhoA inhibitor (Y27632) blocked Ras-induced tumorigenesis even though cells were overexpressed with RhoA, indicating that Ras requires RhoA activation (Figure 3.10C). In sum, these data indicate that GPR54-activating RhoA is sufficient for Ras-induced tumorigenesis.

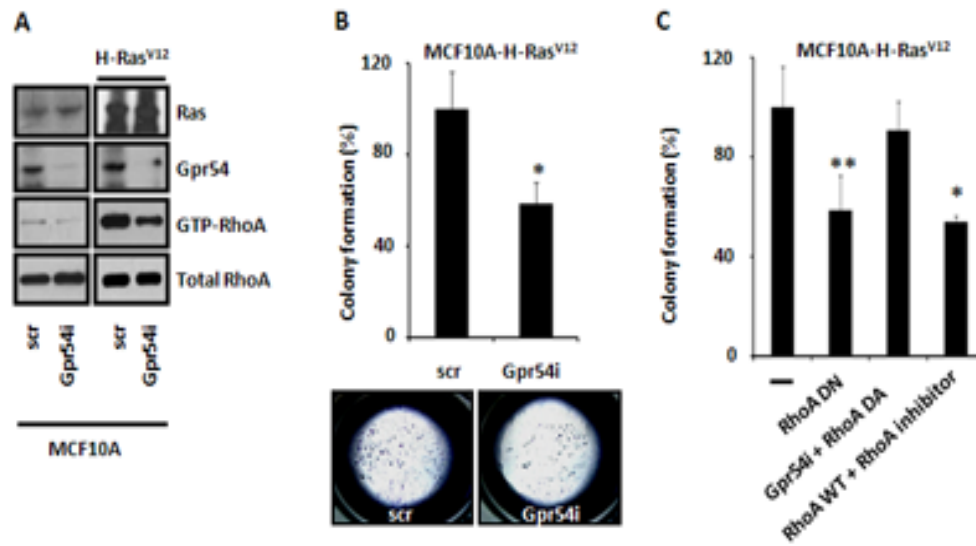


Figure 3.10 Autocrine GPR54 signaling via RhoA regulates Ras-induced tumorigenesis. (A) Ras, GPR54, active RhoA, and total RhoA in MCF10A transfected with the indicatives. (B) Anchorage-independent growth of MCF10A transfected with the indicatives. \*,  $P < 0.05$ . (C) Anchorage-independent growth of MCF10A transfected with the different plasmids and/or treated with Y-27632 (10 $\mu$ mol/L). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

### 3.2 Loss of GPR54 signaling in estrogen receptor-positive breast cancer cells promotes E<sub>2</sub>-independent tumor growth and metastasis

#### 3.2.1 Expression level of KISS1 and GPR54 is high in non-metastatic ER<sup>+</sup> breast cancer cells

The loss of KISS1 expression in metastatic breast cancer cells reversely indicated that non-metastatic breast cancer cells highly expressed KISS1 (Mitchell et al., 2006). Thus, to understand whether non-metastatic breast cancer cells express both KISS1 and GPR54 to achieve the autocrine path, an expression of KISS1 and GPR54 was examined in normal breast epithelial cells (MCF10A), non-metastatic breast cancer cells (MCF7, T47D), and metastatic breast cancer cells (MDA-MB-231, MDA-MB-435). KISS1 mRNA and protein were highly detected in non-metastatic breast cancer cells (MCF7, T47D), reduced in metastatic breast cancer cells (MDA-MB-231, MDA-MB-435), and not shown in normal breast epithelial cells (MCF10A) (Figure 3.11). However, GPR54 mRNA and protein were detected in all cell lines, although its expression level was comparably decreased in metastatic breast cancer cells (MDA-MB-231, MDA-MB-435) (Figure 3.11). Therefore, the endogenous GPR54 signaling might be alive in non-metastatic breast cancer cells.

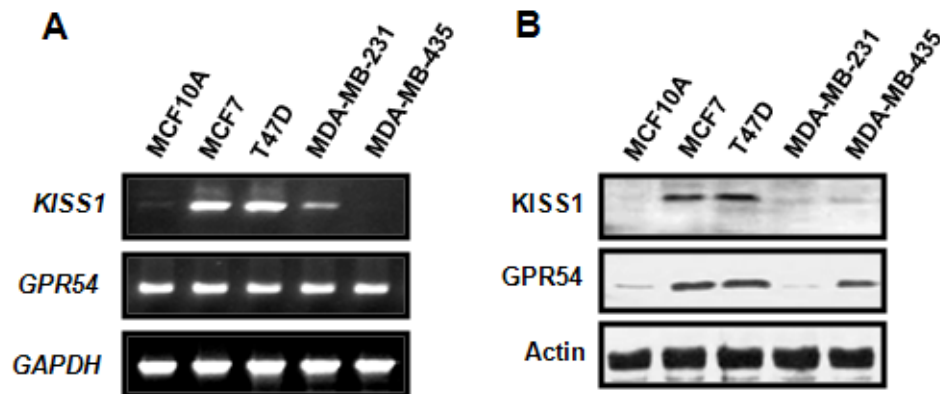


Figure 3.11 Expression pattern of GPR54 and KISS1 in different breast cancer cell lines (MCF7, T47D, MDA-MB-231, and MDA-MB-435) and the immortalized normal breast epithelial cell line (MCF10A). (A) KISS1 and GPR54 mRNA level. GAPDH was used for the internal control. (B) KISS1 and GPR54 protein level. Actin was used for the internal control.

### 3.2.2 Loss of GPR54 signaling in non-metastatic breast tumor cells acquires metastatic cell morphology with an altered expression pattern of EMT and cadherin genes

To examine GPR54 signaling in non-metastatic breast cancer cells, MCF7 cells were stably transfected with constructs containing siRNA sequences for scramble (MCF7-scr), KISS1 (MCF7-KISS1i), or GPR54 (MCF7-GPR54i). Knockdown of either KISS1 or GPR54 expression was confirmed by examining levels of RNA and protein (Figure 3.12A). MCF7-scr was cobblestone-like, but MCF7-KISS1i and GPR54i were spindle-like (Figure 3.12B). Furthermore, when MCF7-GPR54i was transfected with exogenous GPR54, an epithelial morphological shape was recovered (Figure 3.12B).

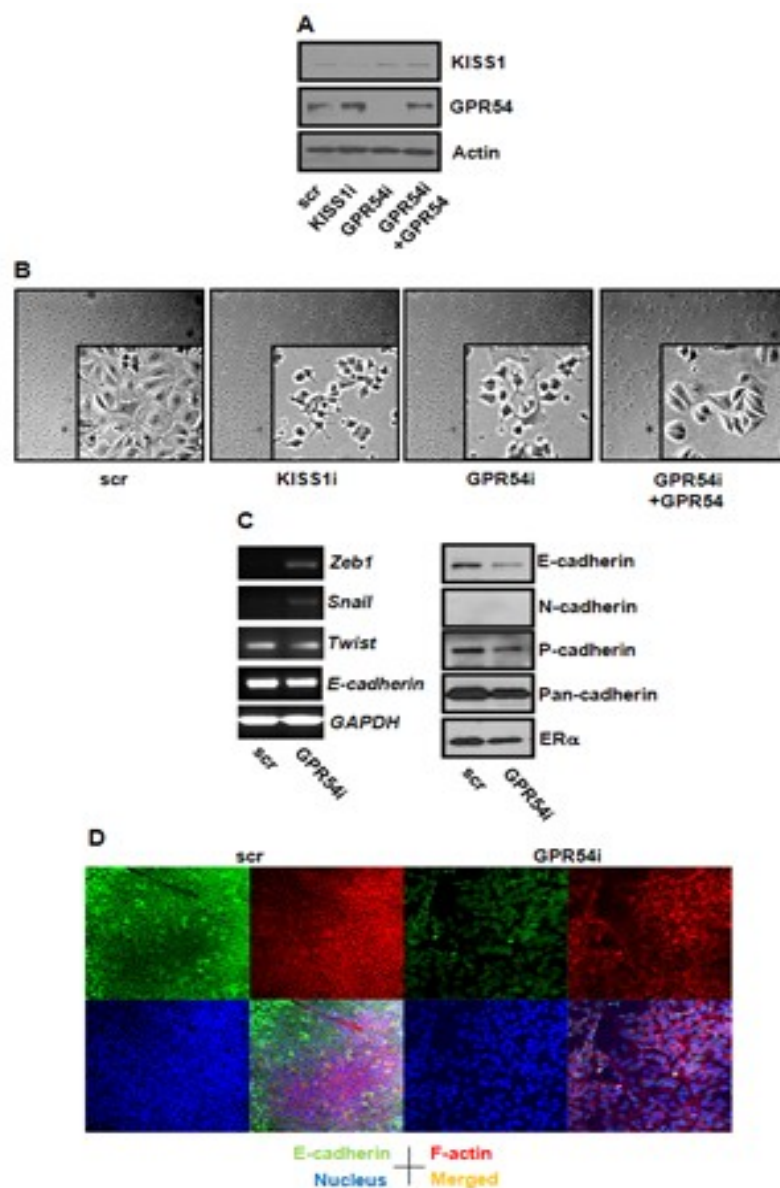


Figure 3.12 Loss of GPR54 signaling and metastatic conversion of cell phenotype in vitro. (A) KISS1 and GPR54 expression in cells. (B) Cell morphologies. (C) RNA and protein expression levels of EMT genes (D) Immunocytochemistry for E-cadherin expression.



As the loss of GPR54 signaling in non-metastatic breast cancer cells caused morphological changes, we further examined whether KISS1/GPR54 signaling affects EMT gene expression and cadherin switch. MCF7-GPR54i, compared to MCF7-scr, showed an increased mRNA level of ZEB1 and SNAI-1 but not TWIST (Figure 3.12C). Likewise, in MCF7-GPR54i, compared to MCF7-scr, E-cadherin (*CDH1*) mRNA level was slightly reduced but N-cadherin (*CDH2*) mRNA level was not detected (Figure 3.12C). Consistently, protein level of E-cadherin was also reduced by GPR54 knockdown (Figure 3.12C and D). Thus our data suggest that the KISS1/GPR54 signaling might maintain the epithelial-like tumor cell feature to block metastasis with a morphological conversion to mesenchymal-like tumor cell.

### 3.2.3 Loss of GPR54 signaling in non-metastatic breast tumor cells gains metastatic cell characteristics

As tumor cell growth is retarded through metastatic progression, we next examined whether the loss of GPR54 signaling affects cell growth. When cell proliferation of MCF7-scr, KISS1i, and GPR54i was examined, the loss of GPR54 signaling decreased cell proliferation by 50% (Figure 3.13A). As EMT increases cell motility, we further performed scratching assays to examine whether the loss of GPR54 signaling affects cell migration ability. Migrated cell number of either MCF7-KISS1i or -GPR54i was increased by about 80% as compared to MCF7-scr (Figure 3.13B). Next, we performed two-chamber assays to examine whether the loss of KISS1/GPR54 signaling affects the invasiveness. Invasion of MCF7-KISS1i or -GPR54i was increased by 100% as

compared to that of MCF7-scr (Figure 3.13C). Furthermore, MCF7-KISS1i or -GPR54i, compared to MCF7-scr, grew much quickly in the anchorage-independent growth assay (Figure 3.13D). In addition, GPR54 reintroduction into MCF7-GPR54i recovered mechanisms altered by the loss of GPR54 signaling (Figure 3.13).

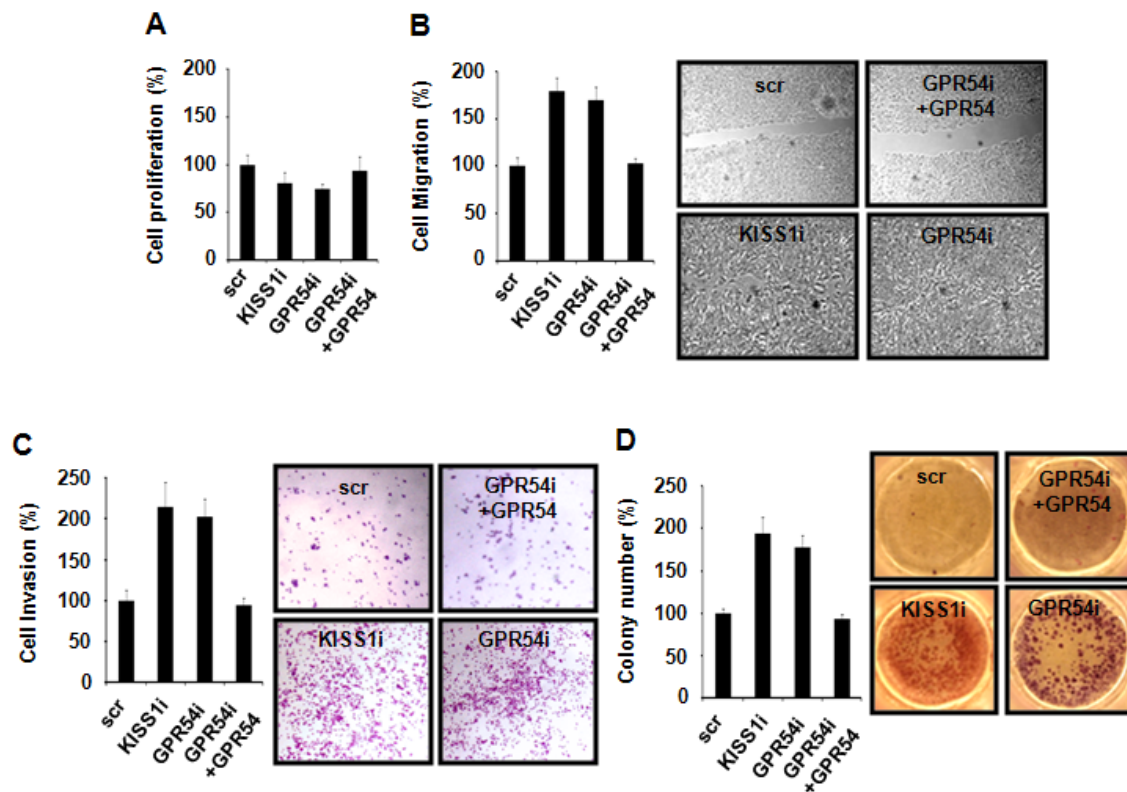


Figure 3.13 Loss of autocrine GPR54 signaling induces metastatic ability. (A) Cell proliferation. (B) Cell migration. (C) Cell invasion. (D) Anchorage-independent growth.

### 3.2.4 Endogenous GPR54 signaling maintains E<sub>2</sub>-dependent growth

Metastatic ER<sup>+</sup> breast cancer cells, albeit retain ER, acquire estrogen insensitivity, thereby they are resistant from endocrine therapy (Anan et al.; Blamey et al., 2010; Broom et al., 2009). As the loss of GPR54 signaling switched non-metastatic ER<sup>+</sup> cells to metastatic, we next investigated whether the loss of GPR54 signaling further caused estrogen insensitivity *in vitro*. MCF7-KISS1i and -GPR54i, compared to MCF7-scr, showed a slight decrease of an expression of estrogen receptor alpha and beta, and did not promote ERBB2 expression (Figure 3.14A). Furthermore, tamoxifen did not affect cell proliferation in MCF7-KISS1i or -GPR54i, although it led to a decrease of MCF7-scr cell proliferation and an increase of cell death (Figure 3.14B).

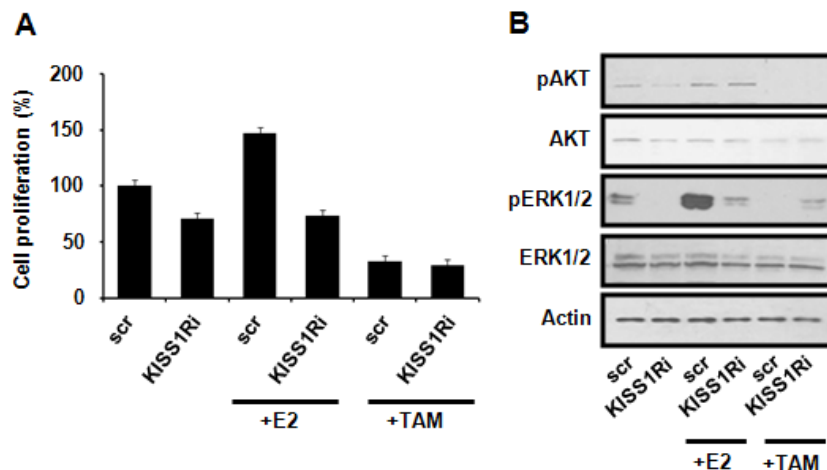


Figure 3.14 Loss of GPR54 signaling affects E<sub>2</sub>-dependent proliferation without obtaining tamoxifen resistance. (A) Cell proliferation. Cells were stimulated with E<sub>2</sub> or tamoxifen for 24hr. (B) Western blot for cell proliferation. As readout, phosphorylation of AKT and ERK1/2 was observed. Actin was used for the internal control.

### 3.2.5 Loss of GPR54 signaling leads to E<sub>2</sub>-independent MCF7 breast tumor growth and metastasis

To understand functions of the endogenous GPR54 signaling in non-metastatic breast cancer in vivo, we performed the xenograft tumor assay where MCF7 cells were orthotopically injected into Nu/Nu mice. Under E<sub>2</sub> stimulation, tumor growth of both MCF7-KISS1i and -GPR54i was slower than that of MCF7-scr, since tumor of either MCF7-KISS1i or -GPR54i was 20% smaller in size than MCF7-scr (Figure 3.15A). While MCF7-scr failed to grow tumor independently of E<sub>2</sub> as consistent with a general notion, both MCF7-KISS1i and -GPR54i did (Figure 3.15A). Thus, our data demonstrate that the loss of autocrine KISS1/GPR54 signaling promotes E<sub>2</sub>-independent growth of ER<sup>+</sup> tumor. H&E staining data further showed that MCF7-KISS1i or -GPR54i, but not MCF7-scr, locally metastasized in the presence or absence of E<sub>2</sub> stimulation (Figure 3.15B), when we examined a boundary of xenograft tumor cohorts from MCF7-scr, MCF7-KISS1i, or -GPR54i.

Next, we performed the experimental metastasis assay to investigate whether the loss of GPR54 signaling also causes distant metastasis. Mice were injected i.v. with MCF7-scr, MCF7-KISS1i or -GPR54i, and pulmonary metastasis was examined 20 days after injection. MCF7-KISS1i or -GPR54i formed metastatic foci at lung, whereas MCF7-scr failed to metastasize (Figure 3.15C). In addition, H&E staining confirmed that metastatic tumor grew at lung (Figure 3.15D). Thus, our in vivo studies suggest that the autocrine GPR54 signaling suppresses ER<sup>+</sup> breast tumor cell dissemination at the primary organ and metastasis to distant organs, in the autocrine manner.

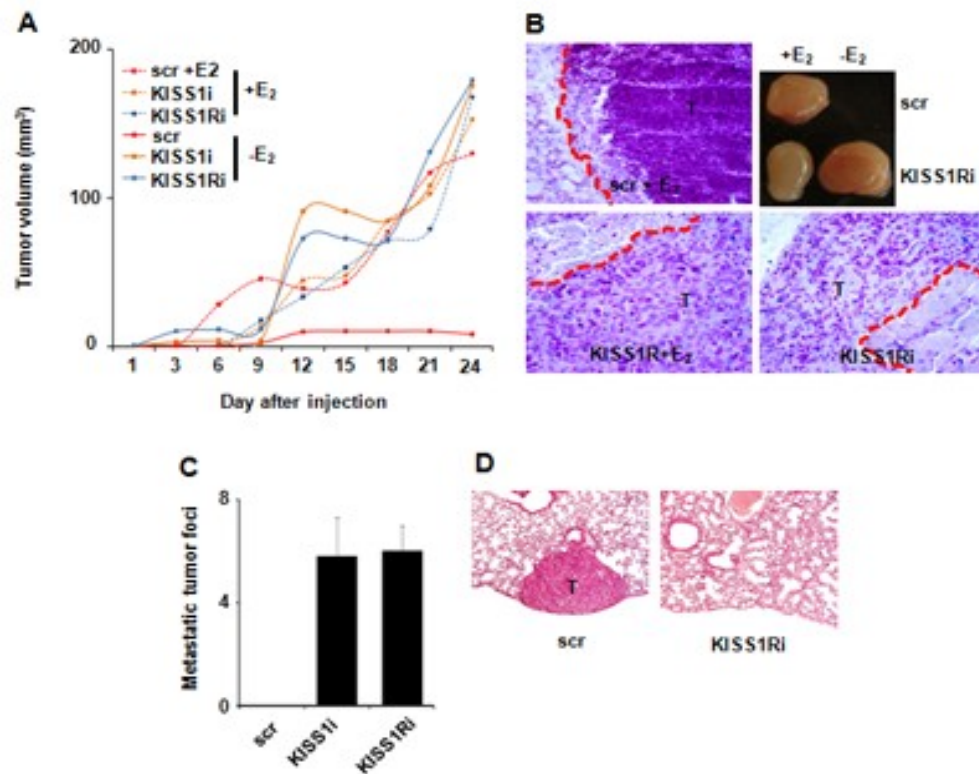


Figure 3.15 Loss of GPR54 causes E<sub>2</sub>-independent tumor growth and metastasis. (A) Xenograft tumor growth. Nu/Nu mice orthotopically injected with the indicated tumor cells were observed until 24 days. (B) Representative images of xenograft tumor cohorts. A top right picture shows the representative tumors. (C) Metastatic foci number. Mice injected i.v. with the indicated tumor cells were sacrificed at 20 day after injection. (D) H&E-staining images of lung tissue. Metastatic tumor foci at lung were counted.

## CHAPTER IV

### DISCUSSION AND CONCLUSIONS

#### 4.1 GPR54 signaling in breast tumorigenesis

GPR54 signaling is known to regulate tumor angiogenesis and metastasis, which was revealed in different model systems (Cho et al., 2009b; Lee and Welch, 1997; Nash et al., 2007; Ohtaki et al., 2001). However, its functional requirement at early stages of cancer development is yet in doubt, although bench-to-bed data are suggestive that GPR54 signaling may affect it at early stages (Dittmer et al., 2008; Hata et al., 2007; Katagiri et al., 2009; Kobel et al., 2008; Marot et al., 2007; Martin et al., 2005; Martins et al., 2008; Mitra et al., 2008; Nagai et al., 2009; Nash and Welch, 2006; Navenot et al., 2005; Nicolle et al., 2007; Schmid et al., 2007; Sousa and Espreafico, 2008). Notably, Gpr54 heterozygosity delayed breast cancer development and metastasis in MMTV-PyMT mouse breast cancer model. In that model, Gpr54 heterozygosity diminished hyperplasia, resulting in the delay of the following events such as tumor formation and lung metastasis. This evidence first provides that GPR54 signaling regulates early steps of tumor development.

In different animal model systems, GPR54 signaling blocked cancer metastases (Lee and Welch, 1997; Nash et al., 2007; Ohtaki et al., 2001). However, animal models for the knowledge of GPR54 signaling in cancer biology have a limitation to understand its function in different stages of tumor progression. Moreover, data from human cancer patients suggest that a role of GPR54 signaling might be not simple (Dittmer et al., 2008;

Hata et al., 2007; Katagiri et al., 2009; Kobel et al., 2008; Marot et al., 2007; Martin et al., 2005; Martins et al., 2008; Mitra et al., 2008; Nagai et al., 2009; Nash and Welch, 2006; Navenot et al., 2005; Nicolle et al., 2007; Schmid et al., 2007; Sousa and Espreafico, 2008). Thus, this study using genetically engineered mouse model gives much knowledge for GPR54 signaling in cancer biology. Although GPR54 signaling is known to inhibit metastasis in different model systems, MMTV-PyMT mouse model system showed that *Gpr54* heterozygosity delayed PyMT-induced mammary tumor latency. Studies using ovariectomized mice suggested that *Gpr54* in breast tissue might not affect post-natal breast development. Thus, *Gpr54* haploinsufficiency seems to be selective for PyMT-induced mammary tumor initiation. Otherwise, GPR54 seems to be required for KISS1-mediated inhibition of tumor cell motility, though Nash et al. remained a question of whether GPR54 is required for KISS1-mediated suppression of metastasis (Nash et al., 2007). Accordingly, it is still unclear whether GPR54 has its own function for cell motility or need a net signaling for kisspeptins-mediated signaling output, as data from primary mouse tumor cell isolated from MMTV-PyMT mouse showed that *Gpr54* heterozygosity decreased cell motility. In addition, *Gpr54* was expressed in metastatic tumors at lung, although it was not detected in aggressive tumor at the primary tumor cohort. Considering a loss of KISS1 linking to cancer malignancy, *Gpr54* signaling might be turned off at lung. Although we suppose that *Gpr54* should have its own function or be activated by other molecules, delayed metastatic tumor growth in *Gpr54* heterozygote could be explained. Recent G-protein coupled receptor (GPCR) studies suggest that GPCRs could crosstalk in terms of an oligomerization.

Otherwise, GPCRs can crosstalk to other transmembrane proteins such as ADAMs, EGFRs and integrins. In addition, kisspeptins are among RF-amide peptides. Thus, it is possible that other RF-amide peptides affect the basal GPR54 signaling in tumor development and metastasis, although previous studies already tested GPR54 binding affinity for various RF-amide peptides (Kotani et al., 2001).

Meanwhile, it is questionable whether GPR54 signaling affects cancer stem cells. Genetic alteration in MMTV-PyMT model may change a predisposition of different breast cell lineages, which can affect particular stages of cancer progression (Qiu et al., 2004; Theodorou et al., 2007; Varticovski et al., 2007). In this study, Gpr54 signaling caused haploinsufficiency for mammary tumor initiation and progression. When we examined an expression pattern of various genes in PyMT/Gpr54<sup>+/+</sup> and PyMT/Gpr54<sup>+/-</sup> mammary glands with tumors at 11 week, Gpr54 heterozygosity affected an expression level of various genes for tumor initiation and development. Thus, it is possible that Gpr54 heterozygosity may alter an expression pattern of endogenous gene sets that can affect tumor initiating-cell propagation. Therefore, a function of GPR54 signaling in cancer stem cells remains to be examined.

Next, GPR54 signaling via RhoA was sufficient for the mammary tumorigenesis. Data showed that GPR54 or RhoA did not induce colony formation in MCF10A, indicating that those are not oncogenic but required for oncogenesis. However, it is still unclear how GPR54-induced RhoA activation affects tumorigenesis in terms of molecular mechanisms. RhoA is known to affect preneoplastic transformation in primary mammary epithelial cells (Zhao et al., 2009) and to function upstream of Cdc42 and



Rac1 to regulate dynamics of cell proliferation and motility (Machacek et al., 2009). Thus, in accordance to my demonstration above, a decrease of a signaling of GPR54 to RhoA might delay tumorigenesis (Du et al., 2004; Khosravi-Far et al., 1996; Narumiya et al., 2009; Qiu et al., 1995; Sachdev et al., 2001; Zhao et al., 2009). To summarize, it is plausible that basal GPR54 signaling to RhoA may lead to the delay of Ras-induced oncogenesis in Gpr54 heterozygosity (Figure 4.1A). However, it is still questionable if GPR54 signaling affects preneoplastic transformation of normal mammary epithelial cells prior to tumorigenic cues. Meanwhile, linking to the previous knowledge of GPR54 signaling, my finding explains, with view of molecular and cellular level, a fact that GPR54 signaling is upregulated in benign tumor and then downregulated in malignant tumor (Figure 4.1B). A basal GPR54 signaling may be sufficient for an oncogenic effect, sustained or enhanced during cancer development, and then turned off by the known mechanisms previously found. Taken together, this study first demonstrates that GPR54 signaling is sufficient for mammary tumorigenesis. However, in order to generalize the finding here, GPR54 signaling in tumorigenesis need to be studied using different model systems.

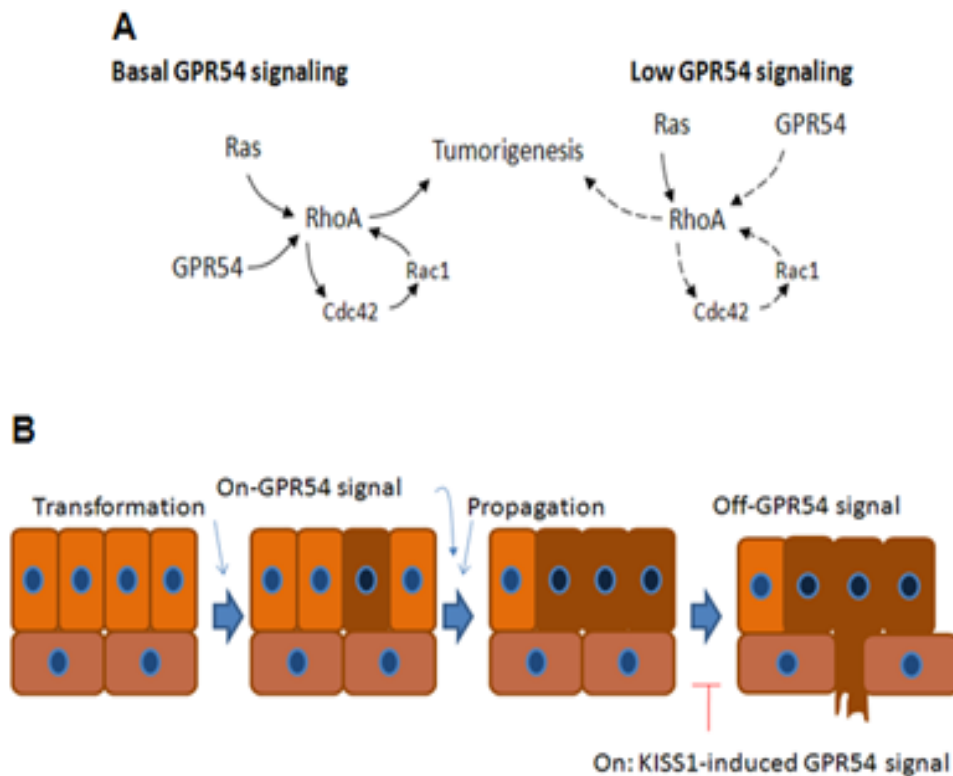


Figure 4.1 Schematic models of GPR54 signaling in cancer development and metastasis. (A) GPR54 basally regulates RhoA-centered dynamics of Rho GTPases. In tumorigenic cues, Ras that is occasionally mutated and abnormally activated functions as the upstream of RhoA. When GPR54 signaling is reduced (or absent), RhoA-centered dynamics of Rho GTPases is disrupted or altered. Thus, tumorigenic signaling is not enough to propagate in time. (B) GPR54 signaling is endogenously working during early stages of tumor development including tumorigenesis, while it is turned off by KISS1 loss during metastatic processes.

## 4.2 Autocrine GPR54 signaling in non-metastatic breast cancer cells

Recent studies have revealed loss of KISS1 gene in metastatic tumor cells, suggesting that GPR54 signaling might follow autocrine path in tumor microenvironment (Lee et al., 1996; Mitchell et al., 2006; Mitchell et al., 2007; Shirasaki et al., 2001; West et al., 1998). The endogenous GPR54 signaling appeared to follow autocrine path to suppress breast cancer metastasis, as KISS1 expression pattern in breast tumor cells is negatively correlated with metastasis (Kostadima et al., 2007; Lee and Welch, 1997; Mitchell et al., 2006; Mitra et al., 2008; Stark et al., 2005). However, there was no loss-of-function study to examine the endogenous GPR54 signaling in non-metastatic breast cancer cells.

Breast tumor metastasis requires a conversion of DCIS to IDC (Jacquemier et al., 2009; Kouros-Mehr et al., 2008; Leonard and Swain, 2004; Lu et al., 2009; Nishidate et al., 2004; Yang et al., 2003). Most human breast cancer cells are ER<sup>+</sup>, although breast cancer is highly heterogeneous (Badve and Nakshatri, 2009; Cleator et al., 2009; Jacquemier et al., 2009; Lopez-Garcia et al., 2010). Recent studies have focused on ER<sup>+</sup> breast tumor cell metastasis, since metastatic tumor cohort also contains ER<sup>+</sup> tumor cells that are known as non-metastatic (Badve and Nakshatri, 2009; Cleator et al., 2009; Han et al., 2006; Lopez-Garcia et al., 2010). Considering a conversion of DCIS to IDC, ER<sup>+</sup> cells appear to experience a global change of molecular and cellular features of ER<sup>+</sup> luminal epithelial tumor cells (Han et al., 2006). With view of molecular and cellular aspect, ER<sup>+</sup> luminal breast tumor cells appear to undergo EMT and, in further, to acquire metastatic ability such as estrogen insensitivity (Belguise et al., 2007; Planas-

Silva and Waltz, 2007; Wang et al., 2007; Ye et al., 2008; Ye et al., 2010). However, molecular mechanisms to maintain non-metastatic features in ER<sup>+</sup> tumor are still unclear.

Morphological change of ER<sup>+</sup> breast cancer cells with the loss of GPR54 signaling suggests that the autocrine GPR54 signaling is endogenously required for maintaining non-metastatic epithelial tumor cell morphology. Interestingly, reintroduction of GPR54 signaling in MCF7 cells stably knocked down with KISS1 or GPR54 rescued cobblestone-like tumor cell shape, whereas KISS1 reintroduction in MDA-MB-231 cells failed to change cell morphology to cobblestone-like (data not shown). It is plausible that reintroduction of GPR54 signaling in metastatic breast cancer cells may not fully recover epithelial tumor cell profiles, since metastatic conversion results in global changes in genomic level (Nishidate et al., 2004). Otherwise, recent studies showed that exogenous GPR54 signaling changed cell morphology with cell growth retardation or apoptotic cell death (Navenot et al., 2009a; Stafford et al., 2002), suggesting that GPR54 signaling might have diverse functions in different biological conditions. Nevertheless, in non-metastatic breast cancer cells, the loss of endogenous GPR54 signaling in autocrine manner altered EMT gene expression and led to an increase of N-cadherin expression. In addition, the loss of GPR54 signaling further altered cell growth, motility, estrogen dependency. Hence, our studies suggest that the autocrine GPR54 signaling is crucial for suppressing ER<sup>+</sup> breast tumor cell dissemination and metastasis (Figure 4.2), which is strongly supported by our *in vivo* studies. In addition, the loss of autocrine GPR54 signaling resulted in an increase of N-cadherin expression with no alteration of

*ESR1* expression. Thus, estrogen-independent tumor growth may be held prior to completion of EMT in ER<sup>+</sup> breast tumor development and metastasis or ER<sup>+</sup> tumor metastasis may not need a complete cadherin switch and/or ER loss. Although GPR54 loss increased an expression of ZEB1 and SNAI-1, known as E-cadherin (CDH1) repressors, *CDH1* mRNA level was not completely repressed. So, certain mechanisms are likely required for complete loss of E-cadherin expression in metastatic breast cancers.

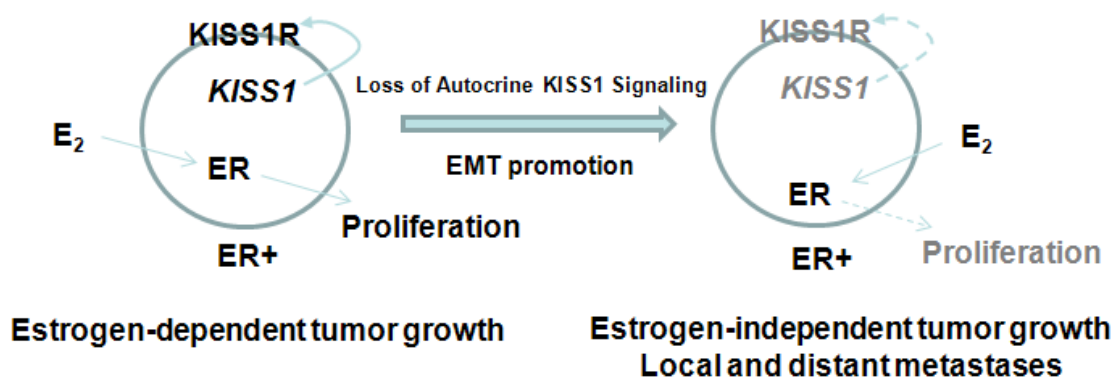


Figure 4.2 Autocrine GPR54 signaling is required for suppressing metastatic turnover. ER<sup>+</sup> breast tumor cells, where autocrine GPR54 signaling is alive, depend on estrogen that has mitogenic function. Hallmarks of a transition of non-metastatic to metastatic cancer are morphological alteration presenting EMT and estrogen-independent growth. Deletion of AP2alpha at chromosome 6 is known to lead to KISS1 loss in metastatic breast cancer cells, thereby autocrine GPR54 signaling is absent in metastatic ER<sup>+</sup> breast cancer cells. Therefore, the endogenous GPR54 signaling in autocrine manner seems to play a role as checkpoint signaling prior to tumor cell dissemination.

### 4.3 Summary

Recent studies for GPR54 signaling in cancer biology have investigated its role in different stages including tumor angiogenesis and distant metastasis. In case of metastasis suppression, exogenous GPR54 signaling has been proposed for cancer therapeutics. However, a role of endogenous GPR54 signaling is still unclear in whole mechanisms of cancer development and metastasis in systematic view. Thus, GPR54 signaling studies require clear information on roles of both endogenous and exogenous GPR54 signaling in early stages together with later stages in different types of cancers. This study focused on the endogenous GPR54 signaling during breast cancer progression including tumorigenesis and, in further, at a conversion stage from non-metastatic ER<sup>+</sup> tumor to metastatic, thereby being expected to help draw a clear map for GPR54 signaling in cancer development and metastasis. Furthermore, this study revealing functions of the endogenous GPR54 signaling would be helpful for cancer therapeutic kisspeptin application.

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